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13. ABSTRACT (Maximum 200 Words) Our overall goal is to determine whether human mammary tumor virus (HMTV) sequences, present as an endogenous retrovirus, are involved in a subset of human breast cancers. In studies completed for first specific aim, samples form a cohort (>200) of breast cancer and appropriate control patients were obtained. In studies under the second specific aim, we identified and sequenced HMTV nucleic acids in breast cancer tissue, control tissue, and blood of patients form our cohorts. Results form a subset of blinded blood samples found that blood from approximately 20% of controls and 70% of breast cancer patients were positive for HMTV sequences. The third specific aim was to construct DNA and cDNA libraries from tissues positive for HMTV proviruses. As this task has been accomplished by others (Pogo and Mason), we are focused on confirming the blinded PCR results in the entire cohort, which is nearly completed. The studies under specific aim four, to express HMTV proteins in an insect cell system, and to characterize the immunological reactions of breast cancer patients and controls against HMTV proteins, are in progress. If a definite link is established, HMTV will provide a target for vaccine development and breast cancer therapy.				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	6
Reportable Outcomes.....	7
Conclusions.....	8
References.....	10
Appendices.....	12

INTRODUCTION:

Sequences with very high similarity (>90%) to mouse mammary tumor virus (MMTV) have been amplified by polymerase chain reaction (PCR) from human breast cancer (BC) tissue (Wang *et al.*, *Cancer Research* **55**: 5173-5179, 1995). The authors of this study presented evidence that the genomic DNA of a subset (~38%) of human breast carcinomas, but not normal tissues, contains sequences that are very highly similar to the MMTV envelope gene (*env*). They suggested the existence of a human mammary tumor virus (HMTV) that is spread by the exogenous route of infection (horizontal transmission). We have PCR amplified sequences highly similar (>95%) to the MMTV *env* gene from human genomic DNA samples, including subsets of both BC tissue and nonBC tissues or blood. A ribonuclease protection assay was used to confirm this result using a non-PCR based technique and to determine that the majority of the PCR positive BC tissues, but none of the PCR negative BC tissues, expressed this sequence at the mRNA level. In addition to mice and humans, we amplified sequences from nonBC genomic DNA of a subset of cats and rhesus macaques distinct from, but highly related to, the MMTV *env*. We also amplified from cat DNA a sequence approximately 90% similar to the MMTV group antigen gene (*gag*). Our results differ from those of Wang and coworkers who, with few exceptions, were able to detect MMTV-like sequences only in breast tumors. Our results indicate that vertebrate species other than mice, including some humans, contain an endogenous retrovirus closely related to MMTV. Our overall goal is to determine whether or not HMTV is involved in a subset of human breast cancers. The first specific aim of this proposal is to recruit and clinically characterize cohorts of breast cancer and appropriate control patients. Various tissues will be obtained from subjects in these cohorts and from archival resources. BC tissues will be staged and classified by standard histological techniques. In studies proposed in the second specific aim, we will identify and sequence HMTV nucleic acids in breast cancer tissue, control tissue, and blood of patients from our cohorts. We will also determine the incidence of HMTV in these various control populations, and compare the sequences of several HMTV genes from different individuals to determine the extent of genetic variability. The third specific aim is to construct DNA or cDNA libraries from tissues positive for HMTV proviruses. These libraries will be screened to identify clones representing the entire HMTV genome. The clones will be sequenced to provide further evidence of the relationship of HMTV to other retroviruses. In studies under specific aim four, we propose to express HMTV proteins in an insect cell system, which allows stable expression of recombinant proteins, and to characterize the immunological reactions of breast cancer patients and controls against HMTV proteins. The proposed studies will establish whether or not HMTV is involved in breast cancer. If a definitive link is established, HMTV will provide a target for vaccine development and breast cancer therapy.

BODY:

Task 1. Recruit and clinically characterize cohorts of breast cancer and appropriate control patients, Months 1-6.

a. We have achieved this goal. We have obtained blood and/or various tissues from about 250 breast cancer and control subjects. A major effort over the last 18 months has been mounting a rigorous blinded "proof-of-concept" trial. We obtained 190 blinded samples, including blood samples from breast cancer patients, healthy controls and a small number of duplicates (10-20).

Task 2. Identify and sequence of HMTV nucleic acids in breast cancer tissue, control tissue, and blood of patients from our cohorts, Months 3-15.

a. The incidence of HMTV in various breast cancer and control populations is being determined. An interim assessment of the results from a subset of blinded blood samples found that blood from approximately 20% of controls and 70% of breast cancer patients were positive for HMTV sequences.

b. Sequences of HMTV from different individuals are being compared to determine the extent of genetic variability. About 30 different HMTV sequences have been obtained.

c. The level of expression of HMTV gene sequences, relative to that of housekeeping genes, by ribonuclease protection assay will be determined. This task is ongoing.

d. The initial publication based on PCR based molecular epidemiology is being written and submitted.

Task 3. Construct and screen genomic DNA or cDNA libraries from tissues positive for HMTV proviruses, Months 15-24.

a. A DNA library has been constructed in Lambda ZAPII from an HMTV positive tumor.

b. The DNA library is being screened by colony hybridization and PCR to identify clones representing the entire HMTV genome. Several clones representing potential hits have been sequenced though we do not yet have a partial or full length sequence from the library. Additional cDNA libraries are being constructed. Because this task was accomplished during the period of this grant by a competitor (Pogo Lab, Liu et al., 2001) and we were aware of a similar result by a collaborator (Dr. Mason - Xu et al., 2004; the PI is the sponsor of Dr. Mason's physician/scientist K08 award from NIH) we focused confirming and validating the PCR results with the entire subset of samples from our cohort.

c. HMTV specific clones will be sequenced to provide further evidence of the relationship of HMTV to other retroviruses and other information. This task is ongoing.

d. Publications based on the complete HMTV sequence will be written and submitted when this task is completed.

Task 4. Characterize immune reactions to HMTV proteins, Months 25-36.

a. HMTV proteins will be expressed in an insect cell system that allows stable expression of recombinant proteins. This task has been initiated though it awaits successful completion of Task 3.

b. Immunoassays using HMTV recombinant proteins will be developed and validated.

c. Immunological reactions of patients with breast cancer and various control subjects will be characterized.

d. Publications based on results from HMTV serological testing will be written and submitted when this task is completed.

KEY RESEARCH ACCOMPLISHMENTS:

- We have obtained blood and various tissues will be obtained from about 250 breast cancer and control subjects. BC tissues were staged and classified by standard histological techniques.
- The incidence of HMTV in various breast cancer and control populations is being determined. Results from the set of blinded blood samples found that blood from approximately 20% of controls and 70% of breast cancer patients were positive for HMTV sequences. We are carefully reassessing these potentially very important results, which are consistent with our hypothesis that HMTV is involved in a large portion of human breast cancer. Representative data from this screen is attached in Fig. 1.
- We have found that minor sequence variations and other sample characteristics greatly affect the ability to amplify HMTV from blood samples. The optimum annealing temperature for each sample is within a very narrow range (± 1 degree C). The optimum temperature varies significantly; for some samples the optimum is 52° C, but for others it is 53°, 54° or 55°. Amplification from tumors is much more robust because of proviral amplification within the tumor. These results partly explain the variation in results among the different research groups investigating HMTV. Representative data from assay optimization is attached in Fig. 2.
- Sequences of HMTV from different individuals are being compared to determine the extent of genetic variability. About 30 different HMTV sequences have been obtained.
- A DNA library has been constructed in Lambda ZAPII from an HMTV positive tumor. Several potential hits have been sequenced, but thus far no positive have been confirmed. We are deriving additional libraries to complete this task to identify clones representing the entire HMTV genome. As noted we reduced the priority of this task as HMTV proviral cloning was accomplished by both a competitor (Liu et al. 2001) and a collaborator (Xu et al., 2004).
- We have initiated studies to express HMTV proteins in an insect cell system that allows stable expression of recombinant proteins. The cell lines have been established and we are inserting the HMTV envelope gene into the appropriate vector for expression. Selection of cell clones that stably express HMTV env should be accomplished in the next month.

REPORTABLE OUTCOMES:

Manuscripts:

*Garry, Robert F., Sara Szabo, Allyson M. Haislip, Sarah E. Nangle, Joshua M. Costin, Vicki Traina-Dorge and Byron E. Crawford, II. Human Mammary Tumor Virus: an update. In: Where We Stand with Breast Cancer Research (N.J. Agnantis Ed.) Synedron Press (Athens, Greece), 15-17, 2001.

*Sara S. Szabo, Allyson M. Haislip and Robert F. Garry. Human and feline proviral sequences highly homologous to selected mouse mammary tumor virus sequences. Accepted, in revision.

Identification of mouse mammary tumor virus related sequences in the human genome. Soble, S.S., Pei, B., Nangle, S., Costin, J. , Crawford, B.E., Haislip, A.M. and Garry, R.F. in preparation.

*attached

Dissertation

Sara Szabo. "Retroviral involvement in human diseases: human and feline proviral sequences highly homologous to selected mouse mammary tumor virus sequences and ultrastructural studies of the human intracisternal A-type particle, type-I, associated with Sjogren's Syndrome." Dissertation approved February 17, 2003 by the Graduate Program in Molecular and Cell Biology in partial fulfillment of the requirements of the Graduate School of Tulane University for the Degree of Doctor of Philosophy

Abstract:

S. Szabo, S., Haislip, A.M., Pai, B. and Garry, R.F. The human contains sequences related to mouse mammary tumor virus Env and LTR. Presented at Tulane Research Day November 2001.

Presentations:

2003	Workshop on receptors and entry of oncogenic viruses. Park City Utah. Invited speaker.
2003	American Society for Virology. Speaker
2002	NIH symposium of the role of viruses in human breast cancer. Bethesda, Maryland. Invited speaker.
2002	Roche Molecular Systems, Alameda, California. Invited speaker.
2001	International Congress of the Hellenic Society for Breast Cancer Research, Athens, Greece. Invited Keynote Speaker.

2000 Viruses and Human Cancer: New Associations. Fred Hutchinson Cancer Center. Seattle, Washington. Invited Symposium Speaker.

2000 Viral Cause of Human Breast Cancer. North Shore University Hospital, Manhasset, New York. Invited Symposium Speaker.

Patents and licenses applied for and/or issued:

*U.S. Patent Application Serial No. 10/018,865; U.S. Patent number 6,670,466 issued December 30, 2003, Title: Human endogenous retrovirus in breast cancer" Inventor: Robert F. Garry.

*attached

Degrees obtained that are supported by this award: Sara Szabo, M.D, Ph.D.

Development of cell lines, tissue or serum repositories:

We have obtained blood and various tissues from about 400 BC and other subjects (including patients with other diseases, such as systemic autoimmune diseases).

Infomatics: not applicable.

Funding applied for based on work supported by this award: none

Employment or research opportunities applied for and/or received: Dr. Szabo was appointed to a faculty level position at Texas Childrens' Hospital.

CONCLUSIONS

A human retrovirus homologous to the mouse mammary tumor virus (MMTV) has been long sought after in breast cancer research (Chopra and Feller, 1969; Dmochowski et al., 1969; Feller et al., 1967; Keydar et al., 1984; Litvinov and Golovkina, 1989). The research supported by the DoD has resulted in strong evidence that sequences closely related to MMTV and endogenous to a subset of humans (circa 20%) are involved in a large subset of human breast cancers (>70%).

The only reported human sequences with very high homology to MMTV have been claimed to represent an exogenous retrovirus. Beatrice Pogo's laboratory detected MMTV-like in human breast cancer samples (Pogo et al., 1997, 1998; Wang et al., 1995, 1998, 2001a,b, 2003), but only a low incidence in blood or tissue from healthy humans. MMTV-like *env* sequences have also been reported in patients who had been diagnosed with both non-Hodgkin lymphoma and breast cancer (Etkind et al., 2000; Wiernik et al., 2000). Witt et al., (2003) found that MMTV-like *env* gene sequences were not detectable in breast cancer tissue of Austrian patients, though Stewart (2003) pointed out several methodological shortcomings of this study. Russian breast cancer patients also express MMTV-related sequences (Kriukova et al., 2002). Ford et al., (2003) reported that MMTV-like gene sequences were amplified in 19 of 45 (42.2%) archival breast cancer biopsy tissues from Caucasian-Australian women, but only 1 of 120 (0.8%) and 0 of 41 breast cancer biopsy tissues from Vietnamese and Vietnamese-Australian women, respectively.

The same sequences were found in only 2 of 111 (1.8%) and 0 of 60 normal (benign) breast tissue samples from Australian and Vietnamese women, respectively. A prevalence of 31-38% positive was found in Argentinian breast cancer samples (Melana et al., 2003). Recently Pogo's laboratory found a higher prevalence (62%) of MMTV-like *env* gene sequences in gestational breast cancer (Wang et al., 2003).

MMTV *env* gene like RNA sequences have been detected by RT-PCR in 66% of breast cancer specimens that were also positive for MMTV *env* gene-like sequences by PRC on genomic DNA (Wang et al., 1998). Additionally, a complete provirus with 94% overall homology to MMTV has been detected in two human breast cancer samples (Liu et al., 2001). FISH was reported to reveal viral integration at several sites in genomic DNA of breast cancer tissue, but not in DNA of normal breast (Wang, 2001a). MMTV uses mouse transferrin receptor 1 for cell entry. Some HMTV had sequence alterations in the putative RBS. Single substitution of one of the amino acids found in an HMTV RBS variant in the RBD of MMTV, Phe(40) to Ser, did not alter species tropism but abolished both virus binding to cells and infectivity (Zhang et al., 2003).

Patients with primary biliary cirrhosis (PBC) have about a four-fold increase in the already high incidence of breast cancer. Previously we showed that PBC patients have both serologic and tissue evidence of retrovirus infection (Mason et al., 1998). Xu et al. (2003) described the identification of viral particles in biliary epithelium by electron microscopy and the cloning of exogenous retroviral nucleotide sequences from patients with primary biliary cirrhosis. The majority of patients with primary biliary cirrhosis have both RT-PCR and immunohistochemistry evidence of human betaretrovirus infection in lymph nodes. Moreover, the viral proteins colocalize to cells demonstrating aberrant autoantigen expression. In vitro, we have found that lymph node homogenates from patients with primary biliary cirrhosis can induce autoantigen expression in normal biliary epithelial cells in coculture. Normal biliary epithelial cells also develop the phenotypic manifestation of primary biliary cirrhosis when cocultivated in serial passage with supernatants containing the human betaretrovirus or MMTV. More recently, the human betaretrovirus was cloned from a cDNA library of a patient with PBC (Xu et al., 2004). Alignment studies performed with characterized MMTV and human breast cancer betaretrovirus amino acid sequences revealed a 93% to 99% identity with the p27 capsid proteins, a 93% to 97% identity with the betaretrovirus envelope proteins, and a 76% to 85% identity with the more variable superantigen proteins. Phylogenetic analysis of known betaretrovirus superantigen proteins showed that the human and murine sequences did not cluster as two distinct species.

The above data and publications are further supportive of the findings reported here, primarily the presence of a human retrovirus with very high sequence homology to MMTV, and also strengthen the arguments for the presence of both endogenous and exogenous forms of HMTV.

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Fig 1.

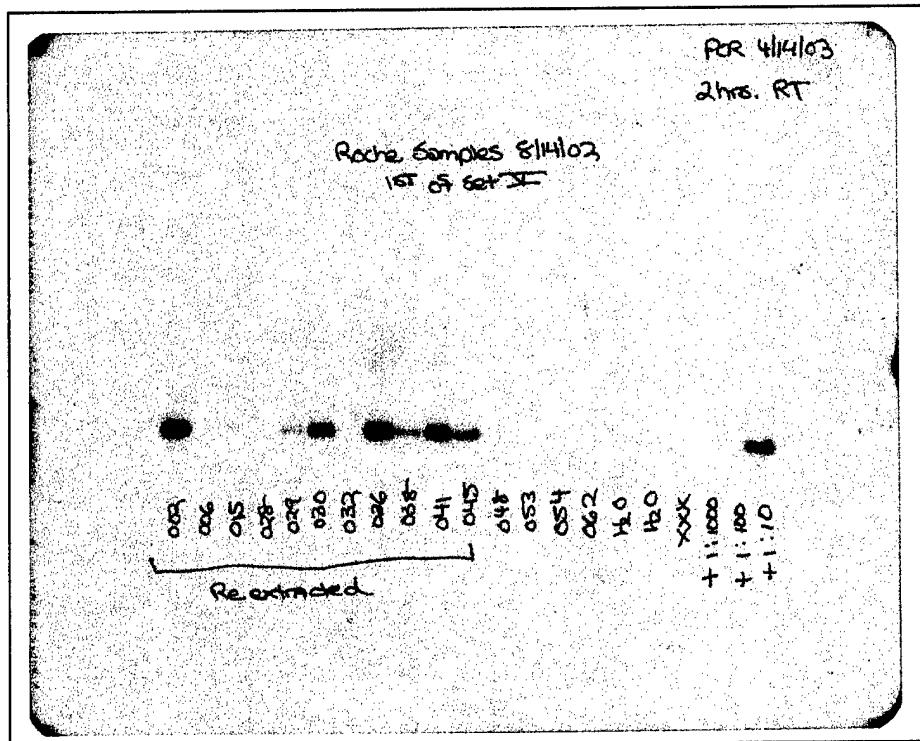
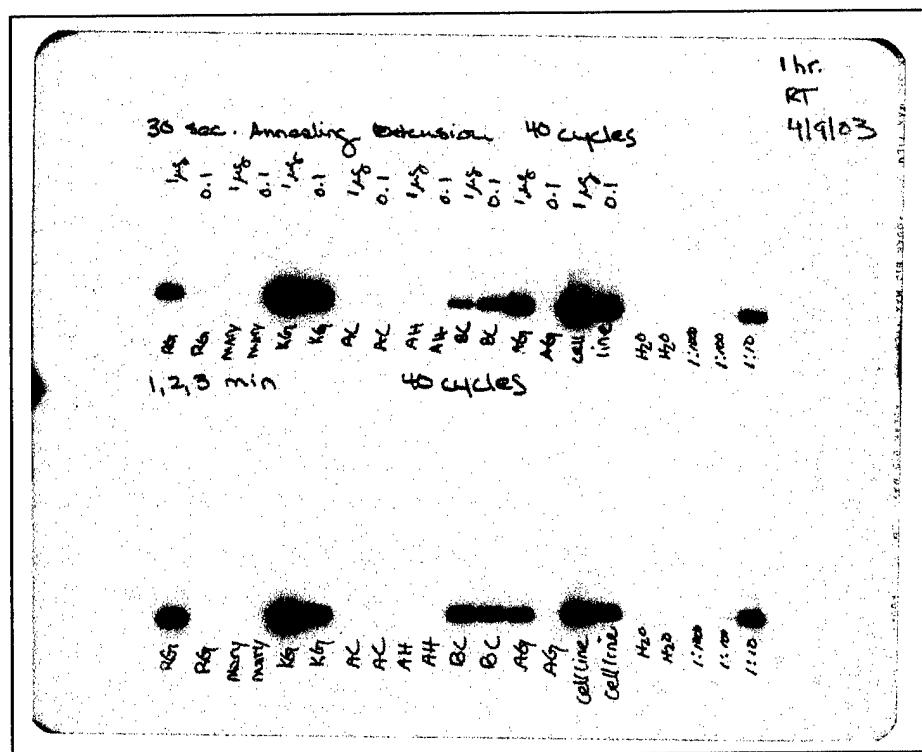


Fig 2

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**HUMAN AND FELINE PROVIRAL SEQUENCES
HIGHLY HOMOLOGOUS TO SELECTED
MOUSE MAMMARY TUMOR VIRUS SEQUENCES**

By

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INTRODUCTION

Ten to twelve percent of women in the United States will develop breast cancer in their lifetime (Ahearne, 1999; Garfinkel, Boring, and Heath, 1994; Robinson E. K., 1999; Sondik, 1994). The incidence rapidly rises during the forth decade of life, becomes substantial before age 50, and continues to increase after menopause, although at a much slower rate. The incidence is high in North America and northern Europe, intermediate in southern Europe and Latin America, and low in Asia and Africa (Ahearne, 1999; Robinson E. K., 1999; Rosai, 1996). People moving from areas of lower incidence to areas of higher incidence gradually acquire an increased risk (Iscovich and Howe, 1998; Winter et al., 1999). Only about 5 % of all breast cancer is thought to arise from inheritance of a mutated gene (Szabo and King, 1997; Welcsh and King, 2001; Welcsh, Owens, and King, 2000; Welcsh, Schubert, and King, 1998). A number of endogenous endocrine factors and environmental factors both in lower and higher socioeconomic classes have been implicated as risk factors in breast cancer development. (Chie et al., 1995; Khuder and Mutgi, 2000; Pukkala and Weiderpass, 1999; Stewart et al., 2000).

Ever since the discovery of the mouse mammary tumor virus (MMTV) (Bittner, 1936; Bittner, 1948; Staff of the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, 1933), lots of efforts have been made to build a human model based on the mouse mammary tumor model, since MMTV is universally considered to be the etiologic agent in the vast majority of murine mammary cancer.

Life cycle of exogenous MMTV

Milk-born exogenous viral particles are transmitted to the suckling offspring; viral particles are taken up in the intestine, and a superantigen-dependent and host immune system-dependent infection is established. Mice are asymptomatic until adulthood, excrete virus into milk and transmit it to their offspring during lactation, and then develop mammary cancer sometime shortly afterwards (Acha-Orbea et al., 1999; Luther and Acha-Orbea, 1996; Luther and Acha-Orbea, 1997; Ross, 2000; Ross et al., 1997). Viral integration into the host genome, as part of the viral life cycle, leads to insertional mutagenesis, which in turn results in

expression of a number of growth factor genes that are normally silent in mammary tissue. Upregulation/dysregulation of these growth factors is thought to be in direct correlation with subsequent tumor development.

The retrovirus: endogenous and exogenous forms

MMTV has both endogenous and exogenous forms. The endogenous forms, derived from rare integration events in the germ line, are transmitted to the offspring in a Mendelian fashion and constitute the Mtv proviruses (Mtv1 – Mtv53) (Kozak et al., 1987). The sequences of common laboratory mouse strains contain on average 3-8 Mtv loci. Most endogenous loci do not produce mature infectious viral particles. Although their *env* gene sequences are often non-functional, their superantigens (SAg) tend to be functional and thus shape the immune system: Mtv mRNA expression can be upregulated through their intact hormone-responsive elements. There are 5-6 Mtv loci that possess intact *env* genes and produce infectious viral particles. Furthermore, two integrated endogenous retroviruses are capable of producing recombinant infectious exogenous particles (Golovkina et al., 1997; Golovkina, Prakash, and Ross, 1996) and coexpression of exogenous and endogenous MMTV RNA in vivo results in production of infectious virions with broadened host range (Golovkina, Jaffe, and Ross, 1994). Thus, even defective endogenous retroviruses have significant biological and potential pathological impact on the host.

Tumorigenesis in mice, different mouse models

In inbred laboratory mouse models, tumor formation occurs at very high percentages of mice, in 100% in some mouse strains, and occurs with relatively short latency, usually before 12 months of age. Numerous transgenic mouse models have been developed (Li, Hively, and Varmus, 2000; Stewart, Pattengale, and Leder, 1984), many of them utilizing MMTV-LTR for targeting transgene expression to the mammary epithelium and allowing stimulation by lactogenic hormones. In reproductively separated feral mouse species, (Callahan et al., 1982; Callahan et al., 1986; Escot, Hogg, and Callahan, 1986; Gallahan et al., 1986; Imai, 1996; Imai et al., 1994; Morris et al., 1977; Schlom et al., 1978) and Asia (Escot, Hogg, and Callahan, 1986; Michalides and Schlom, 1975; Teramoto et al., 1980), tumor formation occurs in low percentages of mice, in certain Asian mouse populations of *Mus musculus* with less than 1% of

incidence, and with relatively long latency, after 18 months of age. Inbreeding of these mice (Imai et al., 1994), resulted in tumor development with an incidence of 80-90% and with tumor latency of less than 1 year, similar to tumorigenesis in inbred laboratory mice.

The distribution of exogenous and endogenous MMTV strains in non-overlapping ranges of three major wild mouse populations is noteworthy. Exogenous viruses have been detected in multiple studies in significant percentages of feral mice. One major species, *Mus domesticus* is the resident species in Western Europe and has been carried to other continents, including North America, via sailing ships. Fifty percent of wild mice in California have been found to carry exogenous viruses. Another species, *Mus musculus* lives in Central and Eastern Europe and most of Asia, and 43% of them carry exogenous viruses (discussed in (Stewart et al., 2000)). Endogenous viruses are carried in a significantly higher number (3-8 per diploid genome) in at least half of the *Mus domesticus* colonies, while at least half of *Mus musculus* carries no endogenous sequences and the rest carries them in low numbers (1-2 per diploid genome). There is significantly less data on a smaller population of a third species, *Mus castaneus*, which is found mostly in South Asia and also has a low copy number of endogenous viruses.

Interestingly, according to a recent analysis (Stewart et al., 2000), the incidence of human breast cancer is highest in areas where *Mus domesticus* is the resident native or introduced species of house mice. In contrast, in areas where *Mus musculus* or *Mus castaneus* are the resident mice, the incidence of human breast cancer is lower. The authors suggest that MMTV-infection should be considered a zoonotic infection, and that MMTV has been transferred to humans from mice. As supportive argument for long time human exposure, they cite ancient documents indicating that mouse fecal pellets have been present in stored grains and thus could have found their way into the human alimentary tract.

MMTV cultured in cells derived from cats and other species

The idea of finding animal models for the investigation of human breast cancer other than those of mice has also been pursued, although with significantly less interest and vigor. MMTV-like viral particles have been isolated from feline cells following serial passage of MMTV obtained from C3H and RIII mouse strains. These variants were shown to be similar to MMTV by immunological and biochemical studies, and had the ability to productively and

efficiently infect *in vivo* cells derived from a number of different hosts, including feline, canine, bat, mink, murine and human cells (Howard and Schlom, 1978). Others have published similar work (Ahmed et al., 1979; Howard et al., 1977; Howard and Schlom, 1978; Howard and Schlom, 1980a; Howard and Schlom, 1980b; Lasfargues et al., 1974; Lasfargues et al., 1976).

An electron microscopic study revealed virus-like particles in 5 out of 11 spontaneous cat mammary tumors. The description of most of these particles matches that of A-type particles, and few budding particles (Feldman and Gross, 1971). C-type particles have been observed in 6 out of 24 feline mammary tumors by another group of researchers (Weijer et al., 1974). Virus-like particles resembling A- and C-type particles were also described in a spontaneous mammary carcinoma of a female rhesus monkey (Chopra and Mason, 1970; Jensen et al., 1970).

None of these reports on spontaneous feline mammary tumors have been followed by further immunological, biochemical or molecular characterization, however. To this date, no further data have been published on MMTV-like viruses or viral sequences either from spontaneous feline mammary tumors, other tissues from cats, or feline cell lines.

Search for a human homologue of MMTV in the last thirty years

A search for a human homologue of MMTV entered its renaissance three decades ago. Scores of studies utilized electron microscopy, biochemical tests to characterize reverse transcriptase activity, various immunological approaches based on antigenic crossreactivity with MMTV, or molecular techniques aiming at the detection of a candidate viral genome, and included investigations of the human genome searching for effects of insertional mutagenesis similar to that of the murine virus. Numerous studies reported MMTV-like viruses or sequences in human breast cancer samples, human mammary carcinoma cell lines, human milk, cyst fluid, breast cancer patients' sera, and peripheral blood monocytes.

Initial ultrastructural and biochemical studies are crude by today's standards, but were promising. B-type and A-type retroviral particles have been visualized by electron microscopy, over thirty years ago, in human milk and human breast cancer (Chopra and Feller, 1969;

Dmochowski, Seman, and Gallager, 1969; Feller, Chopra, and Bepko, 1967; Feller and Chopra, 1968; Feller and Chopra, 1969; Feller and Chopra, 1971; Moore, 1971; Moore, 1974; Moore et al., 1969; Schlom, Spiegelman, and Moore, 1972a). RNA-dependent DNA polymerase activity has been detected and characterized in virus-like particles isolated from human milk (Gerwin et al., 1973; Schlom et al., 1973; Schlom and Spiegelman, 1971a; Schlom and Spiegelman, 1971b; Schlom and Spiegelman, 1973; Schlom, Spiegelman, and Moore, 1971; Schlom, Spiegelman, and Moore, 1972b; Seman and Dmochowski, 1973). Later, enveloped retroviral particles with RT activity were detected in monocytes from breast cancer patients (Al-Sumidaie et al., 1988).

Immunological studies

A plethora of immunological studies followed, searching both for antigens and antibodies that could establish a link between the murine virus (MMTV) and putative human counterpart. Crossreactive antigens were detected on patient samples and human cell lines. Antigenic crossreactivity with the MMTV envelope glycoprotein gp52 was detected by immunohistochemistry on sections of human breast cancer (Keydar et al., 1982; Mesa-Tejada et al., 1978; Mesa-Tejada et al., 1979; Mesa-Tejada et al., 1982; Ohno et al., 1979) in human milk (Litvinov and Golovkina, 1989; Zotter et al., 1980) and in breast cystic fluid (Witkin et al., 1981).

Antigenic crossreactivity with MMTV antigens has been detected in a human breast carcinoma cell line, T47D (Keydar et al., 1979). Retroviral particles in this cell line were visualized by electron microscopy and reverse transcriptase activity was confirmed biochemically (Faff et al., 1992; Faff et al., 1993; Keydar et al., 1984). Crossreactivity was detected between the MMTV gp52 and glycoproteins from the T47D cell line, gp60 and gp68 (Segev et al., 1985). A monoclonal antibody was generated against one of these glycoproteins, gp68, and specific reactivity was reported with pleural fluids only from breast carcinoma patients, but not from other cancer patients (Keydar et al., 1989). Additional mammary carcinoma cell lines have also contained crossreactive antigens using this antibody (Litvinov and Golovkina, 1989).

Antibodies reactive with MMTV antigens have been detected in sera of patients with breast cancer (Day et al., 1981; Dion et al., 1986). It's been reported that in Tunisia, where

there is a high incidence of advanced mammary carcinoma, the incidence of gp52 crossreactivity was high among tumors examined between 1974 and 1984 (Levine et al., 1984). These results have been open to criticism (Dion et al., 1987), however, partly because of apparent *env* gene-related antigenic activity attributed to glycoprotein moieties of human host proteins, and it was suggested that the detected antigenicity was not specific for viral proteins (Hareveni and Lathe, 1990).

Molecular studies

Initial molecular studies turned up a number of candidate sequences. Southern blotting with a number of different MMTV probes under hybridization conditions that allowed partial mismatches yielded MMTV-related sequences from human breast cancer tissue samples (May 1983). Subsequently it was estimated, based on a screen of a human genomic DNA library, that the human genome contains about 50 MMTV-like sequences per haploid genome (Westley and May, 1984). Another genomic DNA library prepared from a human breast cancer cell line, MCF-7, was screened with MMTV probes. The screen yielded a retroviral sequence that had a primer-binding site complementary to lysine tRNA (May and Westley, 1986; May and Westley, 1989). Lysine tRNA is the primer used for reverse transcription of the viral genome both by MMTV and by members of the HERV-K family of human endogenous retroviruses (HERVs). (The designation "K" stands for lysine.) Although finding MMTV-like sequences from a breast cancer cell line looked promising, these sequences now had to be differentiated from other HERV-K sequences.

Further molecular studies collected more data on human endogenous MMTV-like sequences. Numerous papers have described even more numerous copies of elements in the human genome with sequence homology to the MMTV *pol* gene (Sorhaug and Grinde, 1993). Screening of a human phage library yielded *pol* gene sequences with 52% homology to MMTV *pol* gene (Deen and Sweet, 1986). The estimated copy number of such genes varied from 30-40 per haploid genome to 50 per haploid genome. Northern blotting with *gag-pol* gene probes prepared from cloned MMTV-like endogenous viruses detected transcripts of full-length proviral RNAs in human breast cancer cell lines, placentas and some cell lines derived from other malignancies (Franklin et al., 1988). In general, the homology of these sequences to MMTV *gag-pol* sequences has been no more than 60-70%. Many of these HERVs, isolated

from human breast, placenta and normal peripheral blood mononuclear cells, have been named Human MMTV-Like sequences (HML) and have been grouped according to the extent and type of their homology (Andersson et al., 1998; Andersson et al., 1996; Medstrand and Blomberg, 1993; Medstrand, Lindeskog, and Blomberg, 1992; Medstrand et al., 1997). It was estimated that the human genome contains 30-40 copies of endogenous retroviral sequences with 40-68% sequence homology to type B retroviruses and the HERV-K superfamily, and that about 50 copies additional solitary HERV-K-like LTR are present per haploid genome. By this time, claims have been made that previous studies may have detected any of these numerous retroviral sequences or gene products, and that because of their endogenous nature, it would be hard to find an etiologic role for them in human breast cancer, especially in conformity with the Koch's postulates, or it would be difficult to prove such a role, if it existed at all.

The HERV-K10 controversy

Hypotheses and initial evidence started to emerge, however, that endogenous viral sequences may have pathogenic roles in certain diseases (Krieg, Gourley, and Perl, 1992; Larsson, Kato, and Cohen, 1989; Nakagawa and Harrison, 1996; Patience, Wilkinson, and Weiss, 1997; Rasmussen and Clausen, 1997; Weiss et al., 1999; Wilkinson, 1994) and the search among HERVs for MMTV-like sequences has continued. In this pursuit, the HERV-K family has been the focus of much work for a number of reasons. HERV-K10 differs from most other HERVs in possessing open reading frames in all coding regions. It has a high degree of homology to MMTV (Lower et al., 1993; Ono, 1986; Ono et al., 1986), with notable structural similarity of its *env* gene product to that of MMTV. Its LTRs bear all the functional features necessary for viral replication (Lower et al., 1993; Ono, 1986; Tonjes et al., 1996). HERV-K mRNA is transcribed in several teratocarcinoma carcinoma cell lines (Boller et al., 1993; Herbst et al., 1999; Herbst, Sauter, and Mueller-Lantzsch, 1996), and in human peripheral blood mononuclear cells (see above). Of note, observations made on the T47D mammary carcinoma cell line were attributed to endogenous retroviral sequences (Faff et al., 1992) belonging to the HERV-K and RTVL-I family of HERVs (Seifarth et al., 1995). Proviral *pol* gene sequences amplified by RT-PCR from T47D cells showed 65% homology to the type B-related HERV-K10 *pol* gene sequences (Seifarth et al., 1995).

These molecular and immunological data on crossreactivity (see above) pointed in the direction that previous studies detected HERV-K genes and gene products rather than an MMTV-like virus with an etiological role in human breast cancer. One group of researchers, however, was determined to address this controversy by an immunological approach, by investigating the antigenicity of HERV-K and MMTV proteins as detected by viral-specific antibodies. They observed no reactivity between anti-MMTV antibodies and HERV-K envelope proteins, which were represented by a recombinant HERV-K *env* gene product, synthetic peptides, or derived from teratocarcinoma cell lines. Nor was any reactivity found between anti-HERV-K antibodies and MMTV viral proteins, which were derived from a dexamethasone-stimulated MMTV-producing cell line (Vogetseder et al., 1995). These results provide evidence that experiments utilizing immunological crossreactivity between MMTV antigens and putative human MMTV-like viral antigens should not be discredited simply because of the presence of HERV-K sequences in the human genome.

Thus, emerging from the controversy, immunological studies can still be valuable in a search for a MMTV-like virus as an etiological agent in human breast cancer, however attention should be paid to specific viral epitopes and antibodies raised against them. Likewise, a molecular search for such an agent should focus on molecular signatures differentiating the candidate genes and sequences from sequences of other viruses that are not considered to be etiological agents in breast cancer.

Detection of MMTV-like sequences in human breast cancer

With this in mind, in order to circumvent PCR amplification of HERV-K10 sequences from human breast cancer samples, Wang et al. (Wang et al., 1995) selected a region in the MMTV *env* gene that shows low (16%) homology to HERV-K10. They report that amplification was observed in 38.5 % of breast carcinomas, 7 % of fibroadenomas, and in less than 2 % of reduction mammoplasty specimens. Their interpretation was: "These data indicate that 38-40 % of human breast cancers contain gene sequences homologous to the MMTV *env* gene that are absent from other tumors and tissues." These sequences, with 95-99 % sequence homology to the MMTV *env* gene, show the greatest homology to MMTV ever detected. These data make a human homologue of MMTV yet again an attractive candidate as an etiological agent in the development of human breast cancer.

Human genomic molecular markers and molecular links to the mouse model

In addition to a direct search for a putative MMTV-like human virus with gene sequences or gene products of high similarity to MMTV, other studies have investigated the potential molecular and biological effects of a similar putative oncogenic virus in order to determine how good an animal model the MMTV model could be for the human disease. Thus, other research has focused on human genes that can either be used as molecular prognostic markers of human breast cancer, or on the human homologues of mouse genes that become activated by the insertional mutagenic effect of MMTV. Here are some examples.

The *HER-2/neu* oncogene belongs to the epidermal growth factor receptor family. Its overexpression in human breast cancer portends unfavorable prognosis, and anti-*HER-2/neu* treatment is in clinical trials now (Press et al., 2002; Vogel et al., 2001). Mutations and consequent overexpression of *p53* has also been demonstrated in invasive carcinomas (Davidoff et al., 1991a; Davidoff et al., 1991b). Of interest, the human homologue of the murine *int2* is amplified in 15% of breast cancers (Lidereau et al., 1988). The *int-2* gene encodes a basic fibroblast growth factor with the capability of stimulating the growth of epithelial cells. Furthermore, a number of the human homologues of the murine *Wnt* family, like *WNT2*, *WNT3*, *WNT4*, *WNT5A*, *WNT7B* and *WNT10B* are expressed in normal human breast, however *WNT2*, *WNT5A*, *WNT7B* and *WNT10B* genes expressed at elevated levels in proliferative lesions of the breast and in some cell lines (Bui et al., 1997; Huguet et al., 1994).

Additional links between human breast cancer and the mouse model

Yet other less direct links between human breast cancer and the mouse model are related to the immunological reaction of the host to the virus or the immunological state of the host. For example, establishment of MMTV-infection in mice is superantigen-dependent and is accompanied by a superantigen-directed $V\beta$ -specific T-cell deletion. Human T-cells also respond to MMTV-encoded superantigen with $V\beta$ -restriction (Labrecque et al., 1993). The incidence of breast cancer is decreased in immunosuppressed mice (Stewart and Heppner, 1997), and a 44% reduction has also been reported in the incidence of human breast cancer in fully immunosuppressed women following organ transplantation (Stewart et al., 1995).

Additionally, there have been only a handful of reports on laboratory personnel's immune reactivity to MMTV, tested mostly by an assay measuring lymphocyte blastogenesis response, indirect immunofluorescence and ELISA, and occasionally by immunoblotting (Dion et al., 1986). There is a reported case of seroconversion of a documented seronegative laboratory worker with exposure to MMTV and subsequent development of invasive and metastatic ductal carcinoma, which became clinically symptomatic 13 months after the last negative test and 9 months after seroconversion (Poon, Tomana, and Niedermeier, 1983). The mode of transmission or likely transmission in this case was not documented, however.

Correlation of histological features of human breast cancer and those of the murine tumors is a developing field, primarily in conjunction with transgenic mouse studies. Inclusion of studies on feral mice and epidemiological correlation with the human disease may prove to be a useful adjunct, as has been alluded to above. The following work has been pursued along the molecular lines of thoughts in finding direct molecular similarities between MMTV and a putative human counterpart virus, and has also served as a basis for a hypothesis establishing a possible epidemiological link for transmission of this agent from mice to humans.

MATERIALS AND METHODS

Tissue sources, DNA isolation, DNA samples, and controls

Peripheral blood lymphocytes were obtained by the Ficoll-Hypaque purification method from healthy volunteers.

Genomic DNA preparations of various animal and human sources, labeled Z1-Z15 (table I-4.) were kindly provided by Dr. Laura Levy (Tulane University Health Science Center.) Genomic DNA from human breast cancer cell lines (MCF-7, MCF-7M, T47D, MDA, MDA-231, BT, BT20A and HBL-100) and four human breast cancers were a gift from Dr. Steven Hill (Tulane University Health Science Center.)

The H9/RH9 cell line (ATCC CRL-11622) is a T lymphoblastoid cell line, clonal derivative of Hut78, a human T cell line originally derived from a patient with Sezary syndrome. The MSC cell line is an H9-derivative cell-line harboring Human Intracisternal A-type Particle type-1 (HIAP-1), established in Robert F. Garry's laboratory (ATCC VR-1394). The H9 and MSC cell lines were maintained at 37°C in RPMI 1640 supplemented with 10% Serum Plus and 1% penicillin-streptomycin.

Positive controls included mouse genomic DNA (designated as sample Z5) and a cloned MMTV sequence originating from the C3H viral strain (ATCC 45006).

DNA isolation

DNA was isolated from suspended cell cultures and peripheral lymphocytes either by traditional urea-SDS lysis method coupled with phenol-chloroform extraction or by proteinase-K digestion coupled with phenol-chloroform extraction (Ausubel, 1995). For the urea-SDS lysis method, typically, 10^6 - 10^7 cells were resuspended in 150 μ L dH₂O or PBS, then 300 μ L a urea-SDS lysis buffer was added to obtain a concentration of 4.7 M urea, 1.3 % SDS, 0.23M NaCl, 0.67mM EDTA and 6.7mM Tris. Following the addition of 500 μ L phenol, the sample

was sheared using an 18G needle (15 pulls). The procedure was finished by standard phenol/chloroform extraction, followed by ethanol precipitation.

Precautionary steps in handling samples to minimize the chance of DNA cross-contamination

Assembly of polymerase chain reaction for the primary cycle was performed in a laboratory designated specifically for this procedure. No genomic DNA isolation procedures were carried out in that room, no amplification was performed there, and no amplified products were brought back into that room. Specific laboratory rules were set up to minimize the risk of contamination of the PRC reactions. Furthermore, each step was performed in different laboratories or at different designated bench-tops and in different hoods, with appropriate safeguard steps to prevent contamination and with multiple steps of decontamination. The major isolated steps were genomic DNA isolation, assembly of first PCR cycle, amplification, assembly of second PCR cycle, gel electrophoresis and blotting, cloning, PCR selection or of cloned products or selection based on blot hybridization, and sequencing. Disposable supplies were used whenever possible. Template DNA was aliquoted using pipette tips with aerosol filter. Bleach and alcohol were applied regularly to decontaminate pipette barrels, bench surfaces and centrifuge surfaces. As a control, in each set of first and second round PCRs, a template-free reaction ("water control") was included at the end of the reaction set and handled as other reaction tubes that contained template DNA. This approach allowed the detection of both possible contaminants in the PCR mix solution and potential serial carry-over of aerosol contaminants.

Beta-globin gene and ERV2 gene PCR as control for adequacy of human DNA

For control of adequacy of genomic DNA for PCR, we amplified a sequence of the beta globin gene with primers LA1 (5' ACA CAA CTG TGT TCA CTA GC 3') and LA2 (5' CAA CTT CAT CCA CGT TCA CC 3'), as described by Zack *et al.* (Zack et al., 1990). For some experiments we attempted to amplify a sequence of a human endogenous retrovirus, ERV3, as a control for adequacy of genomic DNA for PCR, using primers "ERV3 sense" (5' GAG GCA TAA CTA TAG GAG ATT TGG 3') and "ERV3 antisense" (5' CCT TTC CAA GTC TGA

ACT G 3') (Cohen et al., 1985). Since it would have required significant optimization of amplification by these primers, we abandoned these sequences as controls for amplification.

Polymerase Chain Reaction

Nested PCR on genomic DNA was performed for MMTV *env* and *gag* gene-sequences with primers listed in tables I-5. and I-7. Primer combination for outer and inner primers for first and second round of amplification and the expected size of amplicons are listed in tables I-6. and I-7.

First round of nested PCR for MMTV-like *env*-specific sequences

Concentration of DNA from each genomic DNA source was determined and stock dilutions for PCR amplification were prepared for a typical use of 100 ng DNA in each 200 μ L-amplification tube provided for "Perkin-Elmer 2400" and "Perkin Elmer 9600" Thermocyclers. For assembly of PCR in a total volume of 25 μ L, aliquots of stock PCR buffer was used to obtain a final concentration of 2 mM MgCl₂, 50 mM NaCl, 25 mM Tris (pH 8.2 of 1M stock solution), 1 mg/mL purified bovine serum albumin, and 25mM each of dATP, dTTP, dCTP and dGTP. (For alteration of buffer solution used in PCR optimization see below). *Taq* polymerase was added in concentrations recommended by the manufacturer. Master buffer mixes with appropriate primers were aliquoted before the final step of adding the genomic template DNA to the reaction tubes. Amplification was performed on "Perkin Elmer 2400" and "Perkin Elmer 9600" Thermocyclers in separate laboratories. Our "standard HMTV-*env* amplification" cycle parameters, as designated below, were denaturation at 94°C for 5 minutes, followed by cycling 30 times at 94°C for 1 minute, at 54°C or 50°C for 2 minutes, and at 72°C for 3 minutes, finally held at 72°C for 7 minutes before cooling to 4°C. Touchdown PCR and alterations of parameters for optimization are described below.

PCR parameters altered for optimization of first round of PCR for MMTV-like *env*-specific sequences

Primers and primer pair combinations for nested PCR with primers are listed in tables I-5 and I-6. The primers were designed to MMTV *env* gene regions that show low homology to

human endogenous retroviral sequences. These primers were either identical to or in part based on previously published sequences (Wang et al., 1995).

Different types of alterations of the cycling parameters were as follows. In one approach, various changes of temperature and incubation times were introduced into PRC cycles for each step of annealing, extension and denaturation, and were applied in conjunction with other PCR optimizing approaches. The goals were to make PCR cycling more stringent, and also shorter, primarily to increase specificity and efficacy of amplification. We further wished to determine how the results of PCR with more traditional cycling conditions would compare with the less traditional cycling conditions employed by Wang et al. and by us. Such an analysis could both be of practical value in PCR optimization and may reflect upon unknown attributes of either the genomic sequences themselves or the PCR amplification of these specific sequences.

In a different approach, touchdown PRC was developed for preferential amplification of primer-specific sequences early in the amplification cycle, in order to increase specificity of amplification with reduced background amplification. In our major sets of touchdown PCR, the annealing temperature was gradually dropped with a set slope in consecutive cycles, in a specified range of annealing temperature, for half the number of all cycles. The rationale behind this approach was to start with more stringent annealing temperatures and to gradually reach an unknown, hypothetically more optimal annealing temperature, and thus to enrich products with more specific primer annealing in the early cycles of PCR. In the latter half of the cycles constant annealing temperature were specified in order to effectively amplify sequences that were already amplified in the first half of PCR. Touchdown PCR without introduction of a slope in the annealing temperature was also performed, and other variations and combinations were introduced.

In a third approach, the extension temperature was increased toward the end of the extension phase, with both relatively long and shortened extension times. The theoretical basis of this approach was the idea that extension of amplification by the polymerase could possibly be halted due to secondary conformational constraints in the newly formed, not yet fully extended PCR products; elevation of temperature employed in a hope to denature such secondary structures without denaturation of the annealed and partly extended primers, thereby allowing full extension of the PCR products at higher temperatures.

Reaction buffer composition was also altered in accordance with traditional PCR optimization approaches, like varying the concentrations of magnesium sulfate and / or adding formamide. In separate experiments, additives and detergents, which have a hypothesized role of stabilizing the polymerase during extension, were also added. In some sets of experiments, *Taq* polymerase was substituted with *Deep Vent* polymerase (New England Biolabs) and used with the reaction buffer supplied by the manufacturer. These modifications either did not improve amplification or even resulted in significantly higher background, thus were not used in further experiments.

Second round of nested PCR for MMTV-like *env*-specific sequences

The second round of PCR was also assembled in a total volume of 25 μ L per tube, with $MgCl_2$ of 2 mM in final concentration, *Taq* polymerase in recommended concentrations, and specific second round-primers, similarly to the assembly of first cycle-reactions. Either one μ L of first round-products was added directly to each reaction tube, or a portion of diluted first round-amplicons was added as template for second round. Template-free reactions from the first round, designated as “water control”, were also nested and were handled as the last but one sample of a set in all reaction sets assembled. A “water control” sample for the second round assembly, with no samples added from the first round, constituted the last sample of a set.

Amplification was performed also on Perkin-Elmer 2400 and 9600 Thermocyclers. Our “standard HMTV-amplification” cycle parameters for the second round were denaturation at 94°C for 5 minutes, followed by cycling 42 times at 94°C for 1 minute, 56°C or 50°C for 2 minutes and 72°C for 3 minutes, finally held at 72°C for 7 minutes before cooling to 4°C. PRC amplicons were separated by standard horizontal 1.5 % agarose gel-electrophoresis. Visualization of ethidium bromide-stained gels and documentation was aided by a computer-assisted gel-detection system.

Nested PCR for MMTV-like *gag*-specific sequences

First and second round PRC for MMTV-like *gag*-specific sequences was set up identically to PRC for *env*-specific sequences, only with outer and inner primer pairs specified in table I-7. The primers were designed with the aid of McMolly Tetra software to a portion of

the *gag* sequence of MMTV, C3H viral strain. The selected target sequence had the lowest homology to HERV-K10 and other known endogenous retroviruses. Amplification was performed in Perkin-Elmer 2400 and 9600 Thermocyclers in separate laboratories. Cycling parameters for the first round were denaturation at 94°C for 5 minutes, followed by cycling 30 times at 94°C for 1 minute, 56°C for 2 minutes and 7°C for 2 minutes, finally held at 72°C for 7 minutes before cooling to 4°C. Cycling parameters for the second round were denaturation at 94°C for 5 minutes, followed by cycling 30 times at 94°C for 1 minute, 60°C for 2 minutes and 72°C for 3 minutes, finally held at 72°C for 7 minutes before cooling to 4°C. Products were analyzed by agarose gel-electrophoresis.

Blotting of PCR products for hybridization

PCR products were eluted from agarose gels onto positively charged nylon membranes by a passive downward transfer method in slightly alkaline, denaturing buffer (5x SSC/ 400mM NaOH) for 1.5 hours (Ausubel, 1995). DNA was crosslinked in a UV crosslinking oven at 2,000 kJ. Membranes and gels were visualized under UV light for completeness of transfer.

Random prime labeling of MMTV-*env* and MMTV-*gag* probes for blot hybridization

For preparation of template for *env*-probe, specific sequences were first amplified under our standard PCR conditions on the recombinant plasmid containing the *env* gene of the C3H strain of MMTV, using inner primer pairs ("mmtv-L5" – "mmtv-L3" primer-pair or with the "mmtv-2N" – "mmtv-L3" primer-pair). For a *gag*-probe, specific sequences were amplified on our mouse genomic DNA sample (Z5) under our standard PCR conditions, using an inner primer pair (mgag-3 – mgag-4 primer pair). Gel-purification of the band of expected size was performed using standard gel-purification columns as recommended by the manufacturer (Quiagen). Random prime labeling on the heat-denatured PCR product was achieved by incorporation of ³²P-alpha-dCTP by Klenow fragment, as recommended by the manufacturer (Prime-a-Gene kit, Promega Inc.) Incorporation of the labeled nucleotide was calculated by scintillation counting following standard TCA-precipitation (Ausubel, 1995).

Blot Hybridization of PCR products

Blotted membranes were incubated at 42°C in a roller-tube hybridization oven in prehybridization solution (50 % deionized formamide, 2 mM EDTA, 2 % SDS, 1 mg/mL yeast RNA, 10 % dextrane sulfate, 0.3 M NaCl, 60 mM Tris). For hybridization, the appropriate ^{32}P -labeled probe was added (10^6 cpm) at 48°C for overnight incubation. Two washes were performed with a solution of 2xSSC and 0.1 % SDS at room temperature for 5 minutes, and additional two washes were performed with a solution of 0.2xSSC and 0.1 % SDS at room temperature for 5 minutes. Signals were visualized either by traditional autoradiography or by a Phosphoimager instrument.

Cloning of PCR amplicons

PCR products were ligated into a TA-cloning vector, and competent *E. coli* were transformed according to the manufacturer's protocol (pCR2.1 TOPO Cloning Vector and Kit, Invitrogen). Traditional blue-white differential screening of colonies on X-gal/IPTG agar plates for PCR products inserted into a lacZ-alpha gene fragment, provided on the TA-cloning vector, was not entirely reliable. Therefore colonies were further screened either by PCR, using insert-specific primers, or by replica hybridization. In the latter one, transformed and plated bacterial colonies were replica-blotted onto nylon membranes and then were hybridized with an insert-specific and radioactively labeled probe. Hybridization was performed as described for blot hybridization of PCR products. Colonies, selected either by PCR or replica hybridization, were expanded in 250mL cultures in 1xLB with 100 ug/mL ampicillin. Plasmid-preparation utilized alkaline lysis for disruption of bacterial cell walls and an anion-exchange resin for plasmid binding and elution, and was carried out according to the manufacturer's protocol (MaxiPrep Kit by Quiagen). Before sequencing, purified plasmids were checked for the presence of insert by restriction analysis of the plasmid (EcoRI-digestion releases the PCR insert at two restriction sites immediately flanking the insert).

PCR-screen of colonies for specific inserts

Typically, PCR reactions were assembled in 200 μL -tubes, with a volume of 15 μL , and contained 4 ng/ μL of each primer, 0.25 mM each of the four dNTPs, 2 mM MgCl₂, 50 mM NaCl, 2 mM Tris, 1 mg/mL bovine serum albumin and 0.5 μL of *Taq* polymerase. The

screening primers were identical to the primers used in the second round of PCR. Selected colonies were toothpicked and replica-plated before the addition of PCR mix. Amplification was performed on Perkin-Elmer 2400 and 9600 Thermocyclers with the following cycling parameters: denaturation at 94°C for 5 minutes, cycling 25-30 times at 94°C, 50°C and 72°C for 30 seconds each, and held at 72°C for 7 minutes before cooling to 4°C. Ethidium bromide-stained PCR products were visualized on 1% agarose gels. (For example, see figure I-11.)

DNA sequencing of cloned PCR insert

Sequencing was performed either manually in our laboratory according to the manufacturer's protocols (Sequenase I 2.0, USB), using T7 promoter and M13 reverse primer sequences that flank the inserted PRC product on plasmid pCR2.1, or with an automated sequencer, by custom sequencing. Sequence analysis was performed using McMolly Tetra software.

RESULTS

To insure specificity of isolation of MMTV-like *env* gene sequences in various human and animal genomic DNA samples, a number of steps and approaches were employed. Each step was planned and executed with the goal of detecting sequences highly homologous to MMTV sequences, yet distinguishable from MMTV, and thus specific for the given human or animal species. Nested PCR conditions and primers were designed to conserved MMTV *env* gene regions that show low homology to human endogenous retroviral sequences. Multiple parameters have been altered in attempts to optimize selective PCR amplification of MMTV-homologous sequences in the background of numerous other endogenous retroviral sequences with lower homology. Care was taken to minimize contamination of templates and appropriate control steps were included to detect possible contamination (see Materials and Methods). To ensure MMTV-like homology of amplicons of expected size, amplicons were separated by gel-electrophoresis, blotted onto nylon membranes, and hybridized with an MMTV *env*-specific probe that is internal to the second round PCR primers. Amplicons from positive samples were cloned and sequenced. Definitive conclusions were drawn from, and species-specificity was judged by, comparative sequence analysis of amplicons.

Primer design

MMTV *env*-specific primers were designed with the aim of amplifying a conserved MMTV sequence that has low homology to HERV-K10 or other endogenous human sequences (Wilkinson, 1994). The primers in this work were either identical to, or in part were based on, primers published by Wang et al. (Wang et al., 1995). The sequence spanned by first-round primers mmtv-1 and mmtv-4 encodes a portion of the internal domain of the *env* gene-encoded protein, and is overall highly conserved among MMTV strains with only one glycosylation site encoded. Importantly, it shows low homology (16%) to the *env* gene of HERV-K10 (Wang et al., 1995). Multiple primer pairs located in this region were analyzed in order to obtain better

matches for their respective melting temperatures and to minimize primer-dimer and other primer-artifact formation. Given the constraints in the location of the target sequence, only limited optimization of primers could be achieved in the primer design. Although the mmtv-L5 and mmtv-L3 primers were primarily used as second round primers, they were also utilized in first round amplifications and were nested with primers mmtv-2N and mmtv-3N. The primer pair combinations, along with the expected size of the products, are shown in table I-6.

Amplifications from the first round of PCR

The goal of first-round amplifications was to enrich for possible MMTV-like sequences in the genome of various species other than mouse. Under low stringency amplification conditions, multiple bands have been amplified, with species-specific patterns, from nearly all samples. These bands contain sequences that allow primer binding and amplification with varying efficiency. The majority of the primary amplicons, however, did not amplify with nested primers designed for the MMTV *env* gene. The most prominent bands, in fact, tended to be either shorter or longer than expected from the designs of first round primers, like a duplex of 740 and 800 bps in human samples (figure I-1.) Further patterns from multiple amplifications can be summarized as follows: No prominent and distinct band of expected size was identified unequivocally as MMTV-*env* specific amplicon on any of the samples. Even in mouse samples there were multiple bands of the same intensity, including a band estimated to be of the expected size. In humans (P1, P2, Z1, Z7, Z10 and Z14 in zoo-series), the duplex of 740 and 800 bps seemed to coalesce on some gels, while it appeared to form triplicates on others. There were multiple faint but distinct bands between 250 and 500 bps. The intensely staining coalescing bands above 1,300 bps may represent multimerized PCR artifacts. The rhesus sample (Z15 in zoo-series) showed similarity to human samples, however there was only one dominant band in the region of the duplex of human samples. The nature of these spurious species-specific amplification products has not been investigated. Spurious amplification under our “standard MMTV-amplification conditions” is evident in samples from other, evolutionarily non-related species as well.

Significant spurious amplification in the first rounds called for increasing the stringency. Systematic and stepwise alteration of multiple PCR parameters (see Methods) tended to alter the first round patterns, as expected, both by decreasing or increasing the overall

yield of amplicons, and by decreasing or increasing yield of certain amplicons relative to the band intensity of other amplicons as judged on the electrophoretic gel. However, increasing the stringency did not result in a cleaner and more robust amplification of bands of expected size with significantly reduced background. In fact, while such measures substantially could reduce the background and still support amplification of specific sequences from mouse samples, specific amplification from samples other than mouse was gradually eliminated, as verified by subsequent confirmatory steps. Also as expected, changes in one or both first round-primers resulted in different overall first and second round amplification patterns and changed the efficacy of amplification of MMTV-specific sequences.

Based on the above observations, it was reasonable to evaluate the specificity and efficacy of alterations in various PCR parameters not by first round amplification results but by the subsequent additional steps.

This notion was further supported by comparison of the hybridization signals of first and second round amplification products on PCR blots, and their respective bands on agarose gels under UV light. First round amplicons from some samples other than mouse hybridized only weakly to an MMTV *env*-specific probe (figure I-6.), yet MMTV-like or HMTV-specific sequences were subsequently amplified from these samples by nested PCR and their specificity was confirmed by sequencing.

Amplifications from the second round of PCR

Cycling conditions for second round amplification were also kept at low stringency and with high cycle number at this stage of the optimization. Only those sets of PRC were further analyzed which contained clean "water control" and clean "nested water control" lanes. By agarose gel electrophoresis, most robust amplifications of the band of expected size are consistently seen only in a mouse (Z5) and in one of the cat (Z9) samples (figures I-2., I-3. I-4. and I-5.) In both species, in addition to the band of expected size, some fainter non-specific bands also amplified with the specified primers and under the described PCR conditions. In the other cat sample (normal spleen, Z11) the band of expected size was not only consistently weaker than the Z5 cat sample, but was also present along with numerous relatively prominent non-specific bands. Upon over-exposure, significantly fainter bands of the expected size were visualized in some human samples, like the Z14 sample (figures I-3. and I-4.) In different sets

of PCR, other human samples and the rhesus sample showed equivocal bands upon overexposure of the agarose gel under UV light, which were not comparable to the robust amplification signal seen in the mouse (Z5) or the cat (Z9) sample.

Blot hybridization of second round amplicons

Blot hybridization of second round amplicons (figures I-7., I-8., I-9. and I-10.) revealed some bands that showed weak hybridization signals, which were not visible on stained agarose gels. Conversely, some other bands that seemed to be of the expected size on agarose gels did not hybridize with the MMTV *env*-specific probe. The most important findings are as follows. First, the Z9 cat sample and the Z5 mouse sample always gave comparably robust hybridization signals and above the level of signal intensity of bands in other samples. Because of the nature of the PCR design, no more refined conclusions were drawn regarding the quantity of amplicons. Second, human samples from various sources (like P1, P2, Z7 and Z14, samples) and the rhesus sample (Z15) gave variably strong or weak hybridization signals (figure I-8.). Amplification was variably successful with alternative primer pairs, like with the “mmtv-1N + mmtv-4” pair or the “mmtv-L5 + mmtv-L3” pair for first round of amplification, or with the “mmtv-2N + mmtv-L3” nested primer pair instead of the “mmtv-L5 + mmtv-L3” nested primer pair. Not all PCR reactions from a given individual produced MMTV *env* gene-specific amplicons at all times. Third, in some series, genomic DNA samples from other animals, like those from another cat (Z11), a dog’s spleen (Z4), a chicken’s thymus (Z12) and a snake liver (Z13) showed weaker hybridization signals, either as a single band, or a doublet of bands with one being of the expected size, or showed a tail of radioactive smear starting from a band of expected size. Attempts of cloning specific amplicons from these samples, however, have failed. Fourth, of the three cat samples tested, specific sequences were practically always amplified from the kitten thymus (Z9) sample, which yielded a robust hybridization signal. The Z11 sample, normal spleen, variably yielded a hybridization signal of significantly lower intensity. The Z2 sample, normal kidney from a third cat, has never supported amplification of hybridizing MMTV *env* probe-specific sequences. Fifth, minor differences were observed in the hybridization signal when using the “mmtv-L5 + mmtv-L3” primer pair in contrast to the “mmtv-2N + mmtv-L3” primer pair on the same first round amplicons, nonetheless, the fundamental signal patterns correlated with each other. Sixth, nesting of PCR appears to be a

crucial step for the detection of specific sequences from non-murine samples under the described PCR conditions and with the given primers: PCR blots of first round amplifications tended to show positive signals only in the Z5 mouse and mouse recombinant control lanes, but not in the other lanes, not even in the Z9 cat sample. In contrast, in the second round of PCR, amplification signals of the Z9 cat sample were consistently comparable to those of the Z5 mouse sample.

Sequence analysis

The final conclusions regarding the presence of MMTV *env* gene-specific amplicons in a given sample were based on comparative sequence analysis of cloned PCR products. Percent homology between sequences amplified from different species and from different samples of the same species was determined. The consistency of nucleotide changes in given sequence positions was determined and correlated with species-specificity and inter-species variability. For practical purposes, sequences 100% identical to mouse sequences handled in the lab (sample Z5 and C3H-type *env* sequences) were excluded from further studies as possible contaminants, even if water control and nested water control lanes were free of contaminants by gel electrophoresis and blot hybridization of PCR products. The only claim that can be made based on this work is that from certain samples MMTV *env* gene-like sequences can be amplified, and that these appear to be authentic MMTV-like sequences and not PCR-contaminants from the mouse control samples. It cannot be claimed, however, that other genomic DNA samples do not contain similar sequences, which may have been excluded from the study.

The only two mouse samples ever handled in the laboratory were sequenced in the laboratory as a control for analysis. One sample was mouse genomic DNA from the liver of a Balb/c mouse (sample Z5), not present in the laboratory prior to work on this project. The other one was a cloned MMTV viral genomic DNA sequence (originally isolated from the C3H mouse strain) purchased from NIH for control for this project, and was also absent in the laboratory prior to this work. By sequencing the purchased recombinant control, a difference was detected between the purchased control and the sequence published by the NIH for the cloned MMTV sequence of this strain. This recombinant sequence served not only as a true

control for MMTV sequences handled in the laboratory, but also as a procedural control, for both our PCR and sequencing.

The sequences shown on figure I-12 represent amplicons from genomic DNA of three human individuals, the rhesus sample (Z15) and the kitten thymus sample (Z9). Of the human sequences, "human 1" was isolated from a human breast cancer, "human 2" from a human colon carcinoma (sample Z14) and "human 3" from a human renal cell carcinoma (sample Z7). The two rhesus and two kitten samples represent different amplification reactions. The sequences are overall 95-98 % homologous to the *env* gene sequences of MMTV isolated from the C3H mouse strain (recombinant control, indicated as "mouse" on the figure.) The sequences isolated from different species exhibit signature mutations. In comparison to the mouse sample (C3H of MMTV), point mutational changes occur in the same positions across the board in all three species, are shared only by some of the samples, or occur only in one species or one sample. For example, some of the point mutations in the kitten thymus (Z9) sample also occur in the human renal cell carcinoma sample but not in the other human samples and not in the rhesus sample. Various combinations of shared mutations reveal an emerging species-specific pattern of point mutations. No two species are 100 % identical. None of the presented 600 bp-long PCR products are 100 % identical either to our recombinant MMTV *env*-control, or to MMTV *env* sequences isolated from genomic DNA of the mouse sample (Z5). In the final analysis, these findings, the interspecies variability of mutations and the tendency that mutations are present in non-random nucleotide positions with some conservation across species, indicate that the isolated MMTV-like *env*-related sequences represent authentic species-specific sequences, present in the genomic DNA of species other than mice. This variability of mutations and the pattern of variability are also interpreted as evidence that these sequences are not PCR contaminants from the mouse DNA sources present in the laboratory.

Recognizing both the very high homology of these amplicons to MMTV *env* sequences and the fact that a species-specific pattern is discernable in the point mutations, the argument can be made that it is preferable to incorporate in their name the source of these sequences and refer to them as Human Mammary Tumor Virus (HMTV) and Feline Mammary Tumor Virus (FMTV) sequences. Once more data is available on the rhesus sample, either other amplified genes or other positive rhesus samples, a separate designation for this species may also be

justified. This proposed species-specific designation also recognizes the fact that there are significant differences in the hosts. Plainly, cats are not mice, and humans are neither mice, nor cats. The molecular, genetic and phenotypic differences are significant in these species. Thus some differences should be expected in the interaction between the virus and the host, the natural course of infection, the host's response to viral infection, activation of the virus from dormancy, some aspects of the molecular environment, the phenotypic expression of oncogenicity, and the natural course of tumor development. Recognition of these differences allows better correlation of different animal and human models and allows the acceptance of some of the disparity between the mouse and human mammary tumors already known to us.

Cat *gag* PCR

The high amplification level of *env* genes from the kitten thymus sample (Z9) held the promise that PRC targeting another MMTV-like viral gene in this sample may yield positive results relatively easily. Thus PCR for MMTV *gag*-related sequences was performed following principles and conditions similar to those considered in amplification of *env* sequences, as described in Materials and Methods. A high amplification level was observed by agarose gel electrophoresis and hybridization by a *gag*-specific internal probe. A portion of the amplified *gag* sequence, as compared to the homologous sequence in genomic DNA of a mouse (Z5), is shown in figure I-13. This sequence also shows very high homology (92 %) to the *gag* gene of MMTV. This less ten percent difference in homology between the two genes of the two species (98 % homology for the *env* gene sequence, and 92 % homology - true for a much shorter *gag* gene sequence) is consistent with expected sequence variability between different genes of a virus established in two different species. The above data strengthens the argument that a species-specific feline MMTV-like virus exists, even though only a limited number of cat samples were available for this study and only one of these samples supported amplification of proviral sequences of a proposed FMTV.

DISCUSSION

The data presented here includes amplified sequences with very high (94-98 %) sequence homology to the *env* gene of mouse mammary tumor virus from genomic DNA samples of humans, a rhesus monkey and a cat. Previously identified MMTV-like sequences isolated from human samples have shown a significantly lower (50-70 %) sequence homology, although these experiments targeted polymerase gene regions of putative MMTV-like viruses. Numerous endogenous sequences with variable degree of difference have been identified with this moderate sequence homology, likely belonging to hosts of endogenous retroviral sequences present in the human genome, most of which are defective. These endogenous sequences have been discredited as representatives of a putative MMTV-like agent with etiologic role in the development of human breast cancer. Prior to this work, only one other research group has identified MMTV-like sequences in the human genome with high sequence homology to MMTV (Wang et al., 1995), by targeting the *env* gene for amplification. This work confirmed their finding of very high homology between viral sequences isolated from human and mouse genomic DNA samples. They report that they have isolated these sequences from 38.5 % of breast carcinomas, 7 % of fibroadenomas, and from less than 2 % of reduction mammoplasty specimens. They published these genomic DNA sequences (Wang et al., 1995) and other DNA and RNA sequences isolated subsequently from human breast cancer samples (Pogo et al., 1998; Wang et al., 1998; Wang et al., 2001b) as sequences of a human exogenous MMTV-like virus. In their arguments (Pogo and Holland, 1997; Wang et al., 1995; Wang et al., 2001a), both the exogenous nature of the isolated viral sequences and the high homology to MMTV sequences make this human virus a good etiological candidate for the development of human breast cancer. On one hand it is reasonable to propose that the very high sequence homology is supportive of the notion that viruses represented by these sequences may play a role in the development of human breast cancer similarly to MMTV's widely accepted etiologic role in the development of mouse mammary tumors. On the other hand, data

presented here suggests that dormant or defective endogenous forms of this virus may be present in humans as well, albeit more difficult to isolate than sequences belonging to actively replicating horizontally fully infectious viruses. Additionally presented below is a theoretical consideration of the concept that HTMV should be expected to exist both in exogenous and endogenous forms. A partial deductive analysis is also performed based on what's known about the mouse virus and its tumorigenicity in mice, and how this knowledge would compare to an analogous virus in humans, and how this would fit our current understanding of human breast cancer. Based on further data presented here on MMTV-like samples isolated from cats, this study presents a proposal on a hypothetical link between mice and humans for transmission of MMTV or MMTV-like viruses to humans with subsequent adaptation of the virus the new host.

PCR for MMTV-like *env* sequences, endogenous vs. endogenous sequences

Acceptance or refusal of the hypothesis that a virus is present in purely exogenous forms or also in endogenous forms is a key concept not only for the understanding of the biology of viral infection and viral-host interactions, but also for interpretation of data. For example, considering only those viral forms pathogenic that are of exogenous origin can result in discrediting valid data on a pathogenic endogenous virus and can leave mysterious gaps in our knowledge regarding the biology of an exogenous virus itself. Another example for the importance of understanding of exogenous and endogenous forms is illustrated in the correlation of amplification levels of a target viral sequence (in an experiment designed for quantitative analysis) and the source and nature of the viral sequences. Briefly, if an actively replicating exogenous virus is present in a tissue sample, the copy number of viral genome per cell is expected to be variable, from zero to multiple copies per cell. If a non-defective dormant endogenous virus is present in a sample, ideally and hypothetically at least one full copy of the viral genome is expected per haploid host genome. If a defective endogenous virus is present in the host, the copy number of viral sequences is expected to be constant from cell to cell, although various segments of the viral genome may be deleted or rearranged. If an actively replicating endogenous virus is present in a tissue, a variable copy number of viral genome per cell is expected, ranging from one to multiple, depending on the replicative activity of the virus. Combination of these scenarios may also be possible. Further aspects regarding the

endogenous and exogenous forms and our understanding of the biological and molecular nature of viral host interaction, and resultant pathogenic effects, are discussed below.

PCR optimization for MMTV-like *env* genes, technical considerations

A number of observations regarding the amplification process were intriguing. Most curious of all was the difference in the level of amplification and ease or difficulty of isolation between the mouse (Z5), the kitten thymus (Z9), the other cat samples, the rhesus sample and the various human samples. Furthermore, the background in the amplification reactions, as visualized in agarose gels under UV light, was troubling. It was also peculiar that long and a non-traditional design of cycling parameters was necessary for successful amplification of MMTV-like sequences. The only exceptions to this observation were samples Z5 (mouse) and Z9 (kitten thymus). First traditional technical explanations were sought in optimization attempts of PCR.

A number of factors that could have contributed to suboptimal PCR amplification were reexamined. For one, the constraints in primer design were significant because of the limitations in choosing a target sequence. Given the goal of selective amplification of specific sequences with very high homology to MMTV *env* sequences over endogenous retroviral sequences with lower homology to MMTV, the following had to be considered. Polymerase genes among all retroviruses are highly conserved. Previous experiments have amply proved that differences among the thousands of endogenous *pol* gene sequences are not specific enough to select for a particular retrovirus, like in a quest for an MMTV-like human retrovirus in the work of Blomberg and others (see above.) *Gag* genes encoding "group-specific antigens" tend to be specific, as their names suggest, for a group of viral proteins with similar antigenic characteristics. They may be more unique for a particular class or type of retroviruses than *pol* genes are, but share enough similarities of antigenic sites within a group of retroviruses that lead to antigenic crossreactivity among them. Thus, genes encoding *Gag* proteins show different degrees of homology to one another, but species-specificity should be assessed with caution. *Env* genes of different retroviruses encode a diverse group of viral proteins when compared to one another. Envelope proteins are the viral proteins that would first come in contact with a host cell at the time of infection of that cell. The differences in tissue tropism of different retroviruses are in part reflected in the diversities of their envelope

proteins. Certain portions of *env* genes and envelope proteins are, however, evolutionarily highly conserved, thus ensuring that viral-host contact would lead to efficient interaction with, and subsequently infection of, the targeted host cell. Nonetheless, some minor variability may evolutionarily arise within these conserved regions as well, especially if a virus “jumps” host species, and thus has to interact with host cell surface proteins slightly different from those of the original host of the virus. These mutations may be positively selected for, under adaptational and evolutionary influences, or may be tolerated mutations without negative selection. Such mutations could be used as fingerprints in sequence comparison and analysis of different viruses. Given these considerations, *env* genes of MMTV were compared to *env* genes of other known human retroviruses, including endogenous retroviruses to selection of target sequences (Wang et al., 1995). These primary limitations in target sequence selection, however, pose further limitations in optimal primer selection.

Additional PCR-related factors that could contribute to suboptimal PCR amplification include exhaustion of PCR solution by consumption of factors (like that of dNTPs and salts, or early inactivation or malfunction of *Taq*) – hence the wish to limit the number of cycles and length of steps, which also should help to reduce non-specific background amplification. Additionally, accumulation of non-specific products could be inhibitory by competition with or partial blockage of extension of the sought-after specific products – hence the desire, again, to reduce the background. Furthermore, at given temperatures, the partly amplified products may assume secondary structures by themselves, with each other, or with other non-specific amplicons, which inhibit efficient amplification of specific products. Also, artifacts produced under low stringency conditions may assume structures that would amplify in subsequent cycles even at higher stringency – hence the attempts to alter multiple cycling parameters in various ways.

Furthermore, it is possible that amplification is hindered or blocked in the very first few cycles of PCR by factors inherent to the viral DNA integrated into the host genome. Such hypothesized factors may cause inefficient denaturation of the target sequence, partial annealing of the primers or inefficient extension, ultimately inefficient amplification of target sequences. All these may also contribute to artifact formation and to a vicious circle.

Biological factors

Some of the biological factors that could explain at least some of the above observations are reviewed below. Some of the intriguing observation could be reflections of biological differences between different samples, due to dissimilarities in the nature of the host, of the virus and of the virus-host interactions. One such aspect to consider is the copy number of virus per cell, which factor is dependent on a number of other factors. Another aspect is detection capability of a given number of viruses in different sets of biological and molecular milieu. Different scenarios are presented below, with variations of viral copy number per cell, as determined by the exogenous vs. endogenous nature of the virus, replicationally active vs. dormant vs. defective viral genomes, tissue-specific distribution of viral particles and developmental stage-specific biological activity of the virus, unknown molecular mechanisms that keep MMTV and MMTV-like viruses dormant, and molecular factors and events that bring an end to this dormancy upon activation.

Many of the above factors, endogenous vs. endogenous forms, active vs. dormant vs. defective nature, tissue-specific distribution and developmental stage-specific biological activity of the virus, cannot be independently separated from each other. Let's examine a few exemplary scenarios known about MMTV and MMTV-host interaction, with the thought that the very high sequence homology between MMTV, HMTV and FMTV should help us in experimental design in the investigation of human breast cancer.

In the case of endogenous MMTVs, as we've outlined above, at least one copy per diploid genome is present in every cell. This copy can be of full length, fully functional, producing infectious particles and inducing the development mammary tumors, and in these respects analogous to exogenous viruses. Others are defective, with deletions or other mutations in their genome, producing particles that cannot be transmitted horizontally (are "non-infectious" by themselves), are with or without oncogenic capacities and with or without functional superantigens. A minimal requirement for amplification of MMTV or MMTV-like sequences is the presence of a target sequence, i.e. not be deleted or rearranged such that it would not be amplifiable. When a target sequence is identified in a genomic DNA sample, however, one has to be circumspect in the interpretation of other data to determine whether the sequences belong to an endogenous or exogenous virus, or a virus with both forms. The region of the viral genome to which amplification is targeted may be a critical aspect of the analysis.

Furthermore, the copy number of endogenous viruses per cell may be highly variable. Of feral mice, for example, at least half of *Mus musculus* carries no endogenous sequences and the rest harbor 1-2 copies per diploid genome. On the other hand, at least half of the feral *Mus domesticus* colonies carry 3-8 per copies diploid genome. Common laboratory mouse strains contain on average 28 Mtv loci. Theoretically, with the above premise, humans and cats or other animals may possess one or more copies of endogenous viral sequences, the frequency of which may or may not vary geographically.

In the case of exogenous MMTVs, the viral copy number depends on the tissue type, the neoplastic nature of the tissue, the development stage of the host, and the activity and strain of the virus in conjunction with these factors. In tumors, the copy number of viral genomic DNA is expected to be high depending on the replicative activity of the virus: in addition to an integrated copy of the viral genome in clonally expanded cells, infectious virions may multiply and re-integrate in transformed and yet untransformed cells. The altered genomic, transcriptional and molecular signaling milieu in the neoplastic cells and in their vicinity may aid this process. Naturally, the higher the copy number of all target sequences (all viral copy number), the easier to amplify and isolate the virus. Furthermore, if there is any molecular mechanism that could hinder the availability of viral target sequences for PCR amplification when the virus is integrated in the genomic DNA, freshly reverse transcribed but not yet integrated viral sequences are also expected to be more ready targets for PCR amplification; thus a tissue sample with actively replicating virus can be expected to show higher yield of PCR, whether it is a breast cancer sample or other tissue with a high number of actively replicating virions.

Tissue-specific distribution of the virus adds to the complexity of the evaluation of amplification of a target sequence. In mice, the viral load and the level of gp52 viral antigen is highest in breast samples as compared to other tissues of mice. Lactating breast of mice, which undergoes physiological hyperplasia and expands its cell population on average by 30-fold, also produces a large amount of infectious virions. Infectious, B-type virions of MMTV have also been detected in non-malignant non-lactating breast and salivary glands, although in significantly lower numbers than in breast tumor or non-tumorous lactating breast. In substantially lower numbers MMTV can also be detected in seminal vesicle and prostate tissues. It is difficult to ascertain, however, what the copy number of integrated viruses is in

these tissues, which may not necessarily parallel the level of production of B-type particles. Furthermore, following the establishment of infection, MMTV is believed to spread between lymphocyte subsets even without detectable viremia. The tissue distribution of cells that carry the virus and support spreading of the infection, the timing of spreading of the infection to the breast, as well as the cellular mechanism of viral transfer from one cell to another, are not currently known. It could be interesting to see in years to come which of these factors and aspects of viral-host interactions would apply to humans the other species with MMTV-like viruses.

A further example below also underscores the importance of being mindful of endogenous and exogenous forms of MMTV, HMTV and other related viruses. In evaluating the level of gene-specific amplicons, one needs to take into account the presence, absence or other alterations of the target sequences. In mice, immature (A-type) particles, typically lacking an envelope and containing mostly uncleaved group antigen (gag) proteins, have been detected in B cells of Peyer's patches and draining lymph nodes. Also, several lymphoma and mammary epithelial cell lines express only uncleaved precursor envelope proteins of MMTV and produce only A-type particles. When in hunt for endogenous sequences, these observations certainly cannot be ignored. Interestingly, development of murine lymphoma has been associated with MMTVs mutated in their superantigens. Of note, sequences encoding the superantigen overlap with the *env* gene. How these mutations alter other aspects of viral biology is only partly understood. In general, envelope genes appear to be particularly prone to acquire various mutations that render the gene and gene products defective. In mice, a range of *env*-deletion mutant murine A-type particles (murine IAPs) have been identified and studied extensively (Kakefuda, Roberts, and Suntzeff, 1970; Kuff et al., 1983; Kuff et al., 1986; Kuff and Lueders, 1988; Kuff et al., 1976; Kuff, Lueders, and Scolnick, 1978; Kuff, Smith, and Lueders, 1981; Kuff, Wivel, and Lueders, 1968; Lueders and Kuff, 1989; Lueders, 1976; Lueders, 1987; Lueders, 1991; Lueders, 1995; Lueders et al., 1993a; Lueders et al., 1995; Lueders and Frankel, 1994; Lueders et al., 1993b; Lueders, Grossman, and Fewell, 1989; Lueders and Kuff, 1975; Lueders and Kuff, 1977; Lueders and Kuff, 1979; Lueders and Kuff, 1980; Lueders and Kuff, 1995; Lueders and Mietz, 1986; Lueders, Segal, and Kuff, 1977; Wivel, Lueders, and Kuff, 1973; Yang, Calarco, and Wivel, 1975; Yang and Wivel, 1973; Yang and Wivel, 1974; Yang and Wivel, 1976). Similarly, in humans numerous types of

endogenous viruses have been shown to have their *env* gene lost largely or entirely, or have been shown to carry a variety of other mutations in their genomes (Larsson and Andersson, 1998; Wilkinson, 1994). These and other factors should be considered on an individual basis when designing an experiment.

Based on information gained from MMTV and other human retroviruses and endogenous retroviruses, one should consider the possibility that some tissue types do not support the full maturation of envelope proteins encoded in non-defective endogenous genes, thus producing, for example, A-type particles. This case could constitute an example of how the phenotype of viruses may be dependent on epigenetic host-related factors. Alternatively, transcription of non-defective endogenous genes may be suppressed in some tissue types but not in others, or may be suppressed at different developmental stages. This would be an example for transcription suppression by epigenetic host-related factors.

There are a number of unanswered questions in the MMTV model, which pose even further questions for a putative HMTV model. It is unknown where and how MMTV hides from the initial infection till pregnancy and lactation. It is not known what molecular mechanisms keep either MMTV, or HMTV, dormant, in either the breast or non-breast tissues, exactly what mechanisms play a role and how in activation, either by steroids or other factors, either specific or non-specific for breast tissue. It's not known whether specific modification of genomic DNA at the integrated MMTV or HMTV sequences would possibly play a role in keeping the virus inactive, and whether this molecular mechanism would have other biological roles. One may argue that such a hypothetical specific DNA modification would earmark only viral sequences within the genomic DNA. Such modification would involve either the entire integrated MMTV or HMTV sequence, or only a part of it, such as the LTR, the superantigen region overlapping with the *env* gene region, or other regions of the viral genome. One could also consider whether such modification would alter availability of these modified MMTV-like sequences for conventional PCR-amplification, and how efficiently, if at all, these hurdles produced by epigenetic modification could or could not be overcome by traditional molecular approaches and methods.

Among our observations, we find it interesting that MMTV-like sequences could be amplified from the genomic DNA a human colon cancer (sample Z14) and a human renal cell carcinoma (sample Z7). True, not all assembled PCR samples supported amplification. The

sequences in the above human neoplastic samples of non-breast origin may represent inactive endogenous viral sequences, which may be unmasked in the neoplastic environment and may show limited activity. This may be the result of a bystander effect of the neoplastic process, when the molecular microenvironment changes such that transcription of otherwise silent endogenous viral sequences would be supported and the virus becomes reactivated just in the neoplastic cells, in the neoplastic milieu. Admittedly, the expression of these theoretical endogenous retroviral sequences may fuel already ongoing oncogenesis. Further data is needed to investigate the nature of these and similar sequences in neoplastic tissue of non-breast origin.

Along the same lines of thought, otherwise dormant endogenous viral sequences may be activated in immortalized cell lines, whether of breast cancer origin or of other tissue origin, or may be just simply unmasked or partially unmasked for transcription, even without much replicative activity. The simplest, although not an exclusive, explanation for the finding of amplifiable MMTV-like sequences in a rhesus sample is that we were fortunate to amplify an endogenous viral sequence. However, more data need to be collected to draw further conclusions.

Lessons from the cat samples

The three cat samples are notable for their respective source. The kitten thymus DNA (sample Z9) was isolated from an immunologically active lymphoid organ of a young animal. At the age of this cat, MMTV infection is just being established in mice. It is at this age that suckling mice are either at the end of the first phase of infection by MMTV, after viremia occurred and spread the virus from the Peyer's patches to other organs, or in the second phase, when life-long infection by MMTV is established. In the second phase, all lymphoid organs are involved in a superantigen-dependent immune response. However, not much is known about what happens in non-gut-associated lymphoid organs, specifically in the thymus, in terms of viral replication and further spreading of the virus, and in terms of detection of viral sequences in these organs. Our data on this kitten sample appears to be comparable to phase II. of infection by MMTV of a suckling neonate mouse, although we don't have comparative thymic data on mice. Unfortunately no tissue sample was available for further studies from other organs of this kitten. It is interesting, however, that a significantly weaker amplification signal

was obtained from another lymphoid organ sample, the spleen, from another cat of unknown age, not designated as kitten. Sequences were not isolated from this animal, however. It is also noteworthy that a non-lymphoid organ (kidney) from a cat of unknown age, not labeled as "kitten", did not support even the weakest amplification of MMTV-like sequences. All three samples originated from the same laboratory (LL). Intriguing questions asked about MMTV infection in mice can now be asked about FMTV in cats: exactly how does the infection spread in lymphoid tissue and from organ to organ, how is tissue-specificity determined, where and how does the virus "hide" in dormant phases, what makes and keeps the virus dormant, what activates it, so on and so forth.

Furthermore, as mentioned above, the kitten thymus sample (Z9), with its strongest hybridization signal among the non-murine samples, did not produce hybridizing products in the first round of amplification and yielded a robust signal only after the second round of amplification, in contrast to the mouse genomic DNA sample (Z5), which gave strong signal both in the first and second rounds. These observations may lend themselves to further speculations regarding species-specific host-related differences in case of infection with a highly homologous retrovirus, regarding both the biological nature of the infection and viral-host interactions, and finally the detection of these sequences and interpretation of data.

Human breast cancer as compared to breast cancer in laboratory mice and feral mice

For the development of a working hypothesis for the development of human breast cancer, based on the MMTV mouse model, one should take into account the similarities and differences in cancer development not only between humans and laboratory mice, but also in comparison with feral mice. Many, although not all, inbred laboratory mice used for the study of MMTV-induced oncogenesis carry highly virulent and highly oncogenic strains of MMTV in a fairly homogenous genetic background. Admittedly it is desirable to control various parameters and to reduce numerous variables encountered in an experimental model, and thus it is preferable to work with animals with a stable genetic background and with a strain of virus that efficiently produces the effect to be studied (i.e. here mammary cancer). In such an experimental setting, with continuous inbreeding, more and more virulent viral strains are, or can be, selected for, which selection pressures on either the virus or the mouse are different

from selection pressures in the wild. In the laboratory mouse model, tumor development occurs in up to 100 % of the animals, and the course of tumorigenesis appears to be accelerated with two relatively early peaks of incidence of tumor development, one at 6 months and one at 9 months of age. In sharp contrast, in feral mice, the incidence of spontaneous mammary tumor is much lower, in some areas less than 1%, and tumor usually develops later than 18 months of age.

Regarding humans, in regions of the world with high incidence of breast cancer, which includes the US, 10-12 % of women develop breast cancer, and mostly during the perimenopausal and postmenopausal years. This rate of incidence and the development of cancer at relatively older age better compare to the natural course of cancer development in feral mice than to tumor development in laboratory mice. It has been documented, however, that inbreeding feral mice that carry an infectious exogenous MMTV can result in a viral strain that is virulent, highly tumorigenic, and causes cancer earlier in life. The incidence in this case may reach 80-90 %, and tumor latency shortens to less than 1 year in one study of inbred feral mice from Asia (Imai et al., 1994). In another pedigreed breeding population of feral *Mus cervicolor*, tumor develops with a high incidence and does so between 6 and 14 months of age. In 80% of these animals have a copy of the viral genome is inserted into the *int-1* or *int-2* loci (Escot, Hogg, and Callahan, 1986). As discussed above, even the endogenous viral burden differs in laboratory mice and feral mice. A mouse model used in the development of a model for human breast cancer should be understood both in the context the laboratory and in the wild. Founder effect and other phenomena studied under the aegis of population genetics and epidemiology should also be taken into account.

Furthermore, there are some phenotypic, histologically documented, differences in classic MMTV-induced tumors of mice, chemically induced cancer in mice, the numerous transgenic mouse models published in nearly two decades, and the spectrum of human mammary carcinoma (Cardiff et al., 2000). In fact, minor differences are also seen in the normal development of breast in mice as compared to humans. This area of research is still in its infancy. Some observations are encouraging and better understanding of these similarities and differences should contribute to the development of better models.

The histological / phenotypic differences in normal tissues of mice and humans are presumed to stem from genotypic differences and / or differential epigenetic mechanisms

acting on expression of a given genotype. A carcinogenic agent, whether a virus, chemical carcinogen, or radiation, acts on a species-specific genetic and epigenetic background, which in turn may or may not reverse, halt, or suppress the carcinogenic effect. MMTV, as an oncogenic retrovirus, has a complex interaction with the host. It not only interacts with an individual and not only at the cellular level, from the receptor / viral interaction to insertion in the host's genome, but it also profoundly interferes with the host's immune system, and does so at multiple points. There are numerous potential steps and mechanisms where the host could interfere with the intruder, which counteractive host defense the virus would try to escape. Differences in the host's genetic and epigenetic potential to cope with an oncogenic virus are expected to result in some differences in the attributes of the induced tumor, like the phenotype of tumor and the species-specific natural course of the development, or containment, of the tumor.

Endogenous MMTV in mice and human breast cancer

As discussed above, Stewart *et al.* (Stewart et al., 2000) correlated geographical distribution of different mouse species with different copy numbers of endogenous MMTV and the incidence of human breast cancer in different countries in Europe and Asia. Presented here is a more refined proposal on the same geographical correlation. In their analysis, Stewart et al. (Stewart et al., 2000) correlated the incidence of human breast cancer with a simple distribution map of two main mouse species, namely *Mus musculus* (with a low average copy number of endogenous MMTV) in Central and Eastern Europe, and *Mus domesticus* (with a relatively high average copy number of endogenous MMTV) in Western Europe and North America. There are, however, more detailed maps published in the literature, showing more mouse species and groups of mice related genetically based on genetic similarities of certain enzymes and ribosomal RNA. Of note, Northern Africa, the Iberian Peninsula and Southern France is inhabited not only by *Mus domesticus*, but also by *Mus spretus* (Bonhomme, 1986; Bonhomme et al., 1984; Thaler, 1986). Although it would be interesting to know what the average copy number of endogenous MMTV is in *Mus spretus*, at least we are aware of the fact that the feral mouse population is not uniform in Northern Africa, Spain, Portugal and Southern France, and that these regions constitute the smallest known distribution area of *Mus spretus* (Bonhomme, 1986; Bonhomme et al., 1984). In light of this, it is notable that, the four

countries / regions named by Stewart et al. (Stewart et al., 2000) as the countries with the lowest incidence of human breast cancer in the distribution area of *Mus domesticus* are Sicily, Mallorca, Spain and Portugal. If it turns out that *Mus spretus* carries fewer copies of endogenous MMTV than *Mus domesticus* does, a high incidence of breast cancer would even more tightly correlate with a relatively high copy number of endogenous MMTV in feral mice, with fairly sharp demarcations between areas populated by different mouse species. It would be also intriguing to research what correlation could be made along the North-Eastern shore of the Mediterranean Sea, particularly in Greece, Bulgaria and Turkey, which region is populated by both *Mus domesticus* and *Mus spretoides* species.

Of mice and cats and men. The chain of infection.

These results bring about the hypothesis that cats become infected by MMTV from mouse, and cats transmit the adapted virus to humans.

In this hypothesis, cats acquire / have acquired MMTV from mice primarily through an oral route, by feeding on mice that harbor infectious MMTV strains. Just as exogenous infectious virions may be taken up from milk by lymphocytes in Peyer's patches of suckling mice, theoretically, the gut / mucosa associated lymphoid system of cats could take up infectious virions from mouse feed. Cats could transmit the virus to their offspring orally through milk, or while grooming, from their fur, onto which they smear saliva that could contain virus. Humans in this hypothesis acquire / have acquired MMTV through contact with their infected pets, either mice or cats. Humans, closely adoring their pets and playing with them, may come in direct contact with infectious secretions, like with saliva of their pets licking the owner's hands, or with saliva smeared onto their cats' fur. Infectious secretions on the hand provide a ready route for oral inoculation. Alternatively, humans may get scratched or bitten inadvertently by their pets.

The most highly contagious periods for transmission of the virus from cats and mice to humans are likely to be the animals' newborn, pregnant and lactating periods, when the animals produce and a large amount of infectious virions. These animals may spread infectious virions around the house through drips of milk, saliva drips, and by rubbing their fur on furniture and upholstery. Lactating female animals, protecting their offspring, tend to be even more aggressive than otherwise, thus be more likely to scratch or bite a human intruder.

Of mice and cats and men. Broadened host range of infection.

One may argue that an MMTV strain that adapted to a feline host may more easily establish infection *in vivo* in another host, like humans. First of all, Stewart *et al.* (Stewart et al., 2000) has made the observation that the incidence of human breast cancer is highest in areas where *Mus domesticus* is the resident species of house mouse and suggest that MMTV is introduced into the human population from mice. They correlated the frequently high copy number of endogenous MMTV in this species with the potential of generating exogenous, infectious virions by recombination of endogenous viruses. This latter phenomenon has been clearly documented for MMTV in an experimental setting (Golovkina, Jaffe, and Ross, 1994; Golovkina et al., 1997). The same argument can be applied to cats. Secondly, Howard and Schlom have made the observation host (Howard and Schlom, 1978; Howard and Schlom, 1980b) (also see above) that MMTV passaged through non-murine cell lines, specifically a feline cell line, not only infects other non-murine cell lines easily, but also acquires a broadened host range. In their experiments, these other cell lines were derived from feline, canine, bat, mink and human cells. Thus, one can suggest that recombination either between endogenous viruses, or different strains of endogenous and exogenous viruses, either from feral mice or from infected cats, may yield new strains with a broadened host range, with the capacity to infect and successfully establish infection in other animals and humans. Thus, even if direct contact exposure of men were to be the same both to mice and cats, a cat carrying FTMV may be more infectious for humans than a mouse carrying MMTV.

Of mice and cats and men. Geographical and socioeconomic aspects.

Correlation of data on the incidence of human breast cancer with socioeconomic and geographic parameters has yielded some contradictory conclusions (Chie et al., 1995; Khuder and Mutgi, 2000; Pukkala and Weiderpass, 1999; Stewart et al., 2000). It is a universally accepted view that when people move into a different geographical region, environmental changes are responsible for changes in the risk of developing breast cancer. What environmental factor is the culprit, however, is a matter of debate. For example, some argue that higher living standards pose a higher risk for breast cancer, and to support their arguments they cite the observations that moving from South Asia to the UK or the US increases the risk

of breast cancer. Others counter that the standards of living are relatively high in Taiwan, Japan, Hong Kong and Singapore, and the incidence of human breast cancer is relatively low in these countries. Yet in Africa the risk of breast cancer is still relatively low, although is rising. Proponents of these views also point our that in the proposal Stewart et al. made (Stewart et al., 2000), there is a high incidence of breast cancer in Finland, a country located in the distribution zone of *Mus musculus*. Thus a good correlation between the copy number of endogenous MMTV and the incidence of human breast cancer breaks down in Finland and they claim that the focus should be on high standards of living and associated factors as a risk factors for human breast carcinoma. They acknowledge, nonetheless, that *Mus musculus* species in this region may possibly be mixed with *Mus domesticus*. All parties recognize that more data need to be collected in other parts of Europe, entire Africa, Australia and South-America. Overall, in the above arguments, in the transmission of MMTV-like viruses from mice to humans, there appears to be a missing link that results in discrepancies when correlating different data and theories.

This missing link may be the domestic cat. On one hand, in countries of higher socioeconomic standards, a significant number of women and families have cats as pets. Numerous single women of all ages and families with children have cat pets. Many people have close daily contact with their pets, and likely don't even take note of scratches or occasional bites, let alone spread of virus through saliva as outlined above. Additionally, one could bring up a long list of potential, but currently unknown, factors that would be relevant if such a transmission from cats to humans is proven to take place. Such factor could include the infectious status of a pet, the exogenous and endogenous copy numbers of FMTV in different cat populations, virulence and oncogenicity of the virus and possibly of different viral strains, various aspects of adaptation of FMTV to the human hosts, and other relevant factors.

On the other hand, in countries of lower socioeconomic standards, a possibly significant number of children may play with stray cats, which in turn are likely to feed on mice regularly, thereby providing a short transmission link to humans. The contact may not have to be daily. It is expected that the transfer of established infections to humans would hypothetically correlate with the infectious status of mouse population, susceptibility of cat and human populations, economic standards and social customs.

Of mice and cats and men. Human exposure in ancient times and today.

As discussed by Stewart et al. (Stewart et al., 2000), in support of human exposure to mice, there is documentation that *Mus domesticus* has lived with humans since the beginning of agricultural societies. In support of exposure to MMTV through an oral route, there are ancient documentations on regulatory food standards that specify the limits of the amount of rodent excreta in wheat. Indeed, these documents support the hypothesis that MMTV could have been introduced to humans from mice thousands of years ago.

Additionally, however, cats were domesticated from wild felines in ancient Egypt just about the same time these agricultural societies flourished, exactly with the goal of controlling the rodent population and for protection of grains. Moreover, cats in ancient Egypt were loved, worshiped and thousands of them mummified. The cat-goddess, Bastet, was seen as the goddess of love and fertility. Cats were so highly esteemed that mourning ritual took place when cats died, which included shaving off the owners' eyebrows. Killing a cat was a crime punishable by death, even when cats attacked men. Human exposure to cats may have been just as ancient and regular as to mice, or mouse feces. Furthermore, exposure appears to be more direct and more frequent not only in the past, by also in present times. This is definitely true in present times, especially in societies with high socioeconomic standards, exactly in the countries where breast cancer occurs with the highest incidence. Parenthetically, cat meat has apparently been a culinary treat in some cultures. Other significant culture-specific exposure to cats should be taken into account when a more precise geographical correlation is made with regards to the three host species and the three MMTV-like viruses.

Should these hypotheses above prove to be correct, it appears plausible that enough time has elapsed during human evolution so that endogenous HMTVs could theoretically evolve, whether or not infectious particles would be produced from these endogenous sequences. Nonetheless, the observation that there is an increased risk of breast cancer associated with moving from certain geographical areas to others, coupled with the observation that the incidence of breast cancer tends to be higher in geographical areas where the domestic mouse population harbors more copies of endogenous MMTVs, suggest that there is continual exposure to infectious MMTV and / or FMTV. It is also conceivable that new MMTV and / or FMTV strains infectious for humans may be continuously generated through recombination of endogenous viral sequences.

Of mice and cats and men. Implications of a zoonotic infection.

There are epidemiological implications of our hypothesis on the transmission of MMTV and MMTV-like viruses to humans through FMTV and its feline host, in particular pet cats. If the hypothesis holds true, first preventive actions for the sake of mankind could entail first screening of pet animals (cats, pet mice and hamsters) for the presence of FMTV or other MMTV-like viruses. Second, given the life cycle and biological nature of this virus, development of a vaccine for FMTV or MMTV may prove to be attractive, since such a vaccine could possibly be formulated for oral administration. An oral vaccine could easily be given to pet animals, and if affordable and safe, it could be used in the containment of MMTV in mouse populations. Development of such a vaccine for pets could also provide tremendous amount of experimental data for humans.

Leap from laboratory mice to humans

Finally, the point should be made that for the development of a model for human breast cancer, all the available different models should be utilized, with judicious consideration of their advantages and disadvantages. Major advantages of studies on inbred laboratory mice, versus studied on wild populations of mice (or other animals or humans), are the very well characterized nature of laboratory mouse models, the relative ease of their manipulation and reproducibility, and a number of controllable experimental variables, including genetic variables. The major disadvantages of the MMTV studies in laboratory mice are rooted in the fact that mice are not humans, i.e. that the host-viral interactions are significantly different, a leap from laboratory mice to humans should not be made directly.

Transgenic mouse models offer the advantage of selective manipulation of selected genes on controllable genetic backgrounds. They are excellent research tools, although with the caveat that many of them use MMTV LTR or MMTV promoter and regulatory sequences with the aim of proper tissue targeting of transgenes. The strength of this approach may be the greatest weakness of these models.

Feral mice offer other advantages in a multimodel system of tumorigenesis. Importantly, feral mice are obviously more heterogeneous in their genetic background than inbred laboratory mice are, which is also partly reflected in the development of their tumors.

The relatively low incidence of mammary tumors and the relatively long latency of tumor development in feral mice more closely parallels those of humans, than the incidence and latency of mammary tumorigenesis of laboratory mice do. It is notable, however, that inbreeding of wild mice that carry infectious MMTV results in mice and MMTV strains similar to those in laboratory mouse models; thus the development of highly infectious and highly tumorigenic laboratory mice was not a freak accident, and is reproducible. Studies of MMTV in feral mice can provide a bridge in making a leap from mice models to a human model.

Moreover, with the hypotheses presented above, and FMTV model could make not only a hypothetical link in the study of pathogenesis of HMTV, but also a direct link with epidemiological implications for human populations. Direct studies of human breast cancer should not be neglected, of course, after all humans are neither cats, nor mice.

Furthermore, this hypothesis is congruent with the multistep / multifactorial nature of development of cancer. In the proposal presented above, HMTV and FMTV are very similar to MMTV not only in their sequences but also in their life cycle and biological nature. Therefore, one should also anticipate that the interaction of HMTV with its host is at least as complex as MMTV's interaction with its murine host. Thus people are not expected to be equally susceptible to infection by MMTV, FMTV or HMTV, and likely some people would be resistant to infection. A mechanism for resistance – already well characterized in mice and particular to MMTV in contrast to other conventional viruses – is the host's resistance to action of viral superantigen and resistance to support either establishment or maintenance of infection, at least partially dependent on their immunological make-up. Additionally, a host phylogenetically more developed than the mouse may better armed against an MMTV-like virus than mice are. Perhaps, humans, and maybe cats, have a molecular mechanism that keeps integrated retroviral genomes of HMTV (and FMTV) dormant more efficiently than molecular mechanisms in mice do. These and other potential host-specific factors could significantly influence the natural course and epidemiology of human breast cancer associated with HMTV.

Follow-up on HMTV / human MMTV-like sequences

Further *env* gene sequences have been isolated by PCR with approximately 30 HMTV *env* gene sequences obtained with high sequence homology to MMTV, comparable to the sequence homology presented here. These samples included a number of human breast cancer

tissue, samples from patients with systemic lupus erythematosus (SLE), samples from healthy subjects, and even a sperm sample. The finding of HMTV sequences in SLE patients raises a number of interesting questions on the immunogenicity of HMTV, the answers to some of which may parallel MMTV's interaction with the host, while others may be more similar to those typically encountered with Sjogren's syndrome or other autoimmune diseases. Of note, an A-type retrovirus, the Human Intracisternal A-type Particle, type I (HIAP-I), has been isolated from cocultures of tissue samples from Sjogren's syndrome patients. Classically, and in mice in particular, A-type particles have been thought to be an immature form of B-type retroviral particles. Furthermore, the single most logical explanation for finding HMTV sequences in sperm is that they represent an endogenous form of HMTV in this particular patient.

Furthermore, in Beatrice Pogo's laboratory, MMTV *env* gene like RNA sequences have been detected by RT-PCR in 66% of breast cancer specimens that were also positive for MMTV *env* gene-like sequences by PRC on genomic DNA (Wang et al., 1998). Additionally, a complete provirus with 94% overall homology to MMTV has been detected in two human breast cancer samples. FISH was reported to reveal viral integration at several sites in genomic DNA of breast cancer tissue, but not in DNA of normal breast (Wang et al., 1999 ARC Mtg. Abstracts 2933 and 2944) (Wang, 1999). MMTV uses mouse transferrin receptor 1 for cell entry. Some HMTV had sequence alterations in the putative RBS. Single substitution of one of the amino acids found in an HMTV RBS variant in the RBD of MMTV, Phe(40) to Ser, did not alter species tropism but abolished both virus binding to cells and infectivity (Zhang et al., 2003).

MMTV-like *env* sequences have also been reported in patients who had been diagnosed with both non-Hodgkin lymphoma and breast cancer (Etkind et al., 2000; Wiernik et al., 2000). MMTV-like LTR sequences have also been detected in human breast cancer samples both in our laboratory and in Beatrice Pogo's laboratory (Wang et al., 2001a; Wang et al., 2001b). Witt et al., (2003) found that MMTV--like *env* gene sequences were not detectable in breast cancer tissue of Austrian patients, though Stewart (2003) pointed out several methodological shortcomings of this study. Ford et al., (2003) reported that MMTV-like gene sequences were amplified in 19 of 45 (42.2%) archival breast cancer biopsy tissues from Caucasian-Australian women, but only 1 of 120 (0.8%) and 0 of 41 breast cancer biopsy tissues from Vietnamese

and Vietnamese-Australian women, respectively. The same sequences were found in only 2 of 111 (1.8%) and 0 of 60 normal (benign) breast tissue samples from Australian and Vietnamese women, respectively. A prevalence of 31-38% positive was found in Argentinian breast cancer samples (Melana et al., 2003), and recently Pogo's laboratory found a higher prevalence (62%) of MMTV-like env gene sequences in gestational breast cancer (Wang et al., 2003).

Patients with primary biliary cirrhosis (PBC) have about a four fold increase in the already high incidence of breast cancer. PBC patients have both serologic and tissue evidence of retrovirus infection (Mason et al., 1998). Xu et al. (2003) described the identification of viral particles in biliary epithelium by electron microscopy and the cloning of exogenous retroviral nucleotide sequences from patients with primary biliary cirrhosis. The majority of patients with primary biliary cirrhosis have both RT-PCR and immunohistochemistry evidence of human betaretrovirus infection in lymph nodes. Moreover, the viral proteins colocalize to cells demonstrating aberrant autoantigen expression. In vitro, we have found that lymph node homogenates from patients with primary biliary cirrhosis can induce autoantigen expression in normal biliary epithelial cells in coculture. Normal biliary epithelial cells also develop the phenotypic manifestation of primary biliary cirrhosis when cocultivated in serial passage with supernatants containing the human betaretrovirus or MMTV. More recently, the human betaretrovirus was cloned from a cDNA library of a patient with PBC. Alignment studies performed with characterized MMTV and human breast cancer betaretrovirus amino acid sequences revealed a 93% to 99% identity with the p27 capsid proteins, a 93% to 97% identity with the betaretrovirus envelope proteins, and a 76% to 85% identity with the more variable superantigen proteins. Phylogenetic analysis of known betaretrovirus superantigen proteins showed that the human and murine sequences did not cluster as two distinct species.

The above data and publications are further supportive of the findings reported here, primarily the presence of a human retrovirus with very high sequence homology to MMTV, and also strengthen the arguments for the presence of both endogenous and exogenous forms of HMTV.

CONCLUSIONS

A human retrovirus homologous to the mouse mammary tumor virus (MMTV) has been long sought after in breast cancer research. The only reported human sequences with very high homology to MMTV have been claimed to represent an exogenous retrovirus. Presented here are PCR-amplified sequences with very high homology to the envelope (*env*) gene of MMTV from genomic DNA isolated from human breast cancer and non-breast cancer samples, a rhesus monkey and a cat sample, and subsequently verified the authenticity of amplicons by sequencing. Additionally presented here is a *gag* sequence with very high homology to the MMTV *gag* gene, isolated from a cat sample. A proposal is made that these MMTV-like viruses are highly similar in their biological nature to the mouse virus, that they have both endogenous and exogenous forms, that the host species be reflected in their names and thus be called human mammary tumor virus (HMTV) when isolated from humans and feline mammary tumor virus (FMTV) when present in cats. This designation is also preferable to reflect differences in the host, primarily in investigations of host-viral interactions and tumorigenicity of the virus in the human host.

Additionally shown here is the first direct molecular evidence for the existence of a feline retrovirus with very high homology to MMTV. These viral sequences were isolated from genomic DNA of a cat, with very high homology to both the *env* and *gag* genes of MMTV. Based on the isolation of cat *env* and *gag* gene sequences, an additional hypothesis is presented here detailing that cats may have acquired MMTV from mice, and that humans may acquire the virus through contact with their infected pets, either mice or cats. In accordance with data on cultured MMTV, it is plausible that *in vivo* recombination in the animal host between endogenous MMTVs, or between endogenous and exogenous MMTVs, may produce new infectious viruses with a broadened host range, and thereby pose a threat to humans. Furthermore, contact with infected pet cats may correlate with increased incidence of breast cancer both in wealthier nations and in countries with lower standards of living, albeit for different reasons. This hypothesis carries direct implications for public health and prevention of a zoonotic infection.

TABLES AND FIGURES

Table I-4. Designation code of samples in our "Zoo Set" with respect to the origin of genomic DNA samples.

<u>Sample ID</u>	sample source
Z1	human squamous cell carcinoma EE1
Z2	cat 931 normal kidney
Z3	lambda 25-1 cloned DNA
Z4	dog spleen
Z5	mouse, Balb/c liver
Z6	sea urchin
Z7	human renal cell carcinoma
Z8	rat embryo fibroblasts (REF cells)
Z9	normal kitten thymus
Z10	human sperm
Z11	cat 1325 normal spleen
Z12	chicken thymus
Z13	python liver
Z14	human colon CA
Z15	Rhesus macaque

Table I-5. Sequences of MMTV *env* primers:

Primer designation	MMTV <i>env</i> primers
mmtv-1	CCT CAC TGC CAG ATC (sense)
mmtv-1N	CCT CAC TGC CAG ATC GCC T (sense)
mmtv-L5	CCA GAT CGC CTT TAA GAA GG (sense)
mmtv-2	TAC ATC TGC CTG TGT TAC (sense)
mmtv-2N	CCT ACA TCT GCC TGT GTT AC (sense)
mmtv-L3	TAC AGG TAG CAG CAC GTA TG (antisense)
mmtv-4	GAA TCG CTT GGC TCG (antisense)
mmtv-3N	CTC CGG CGG TAT GCA CGA CG (antisense)

Table I-6. Target size of amplicons with various *env* primer combinations.

(Most commonly used combinations are indicated in bold)

forward primer	reverse primer	size of the expected product	size of the expected product without primers
<u>Outer primer pairs:</u>			
mmtv-1	mmtv-4	686	656
mmtv-1N	mmtv-4	686	656
mmtv-L5	mmtv-4	679	644
mmtv-2N	mmtv-4	691	656
mmtv-1N	mmtv-L3	604	565
<u>Inner primer pairs:</u>			
mmtv-L5	mmtv-L3	594	554
mmtv-2	mmtv-L3	190	152
mmtv-2N	mmtv-L3	192	154
mmtv-L5	mmtv-3N	572	532
mmtv-2N	mmtv-3N	183	143

Table I-7.

Sequences of MMTV *gag* primers:

Primer designation	MMTV <i>gag</i> primers
mgag1	CAG GCA AGC GAA AGG GCA AG (sense)
mgag2	GCA TGG AGA GCA ATT CCG CC (sense)
mgag3	CAA TTC CGC CTC CTG GAG TT (sense)
mgag4	ATA CCC TGA ACA ACT GCG GG (antisense)
mgag5	AAG CCT TCT GAG CCT CGT TC (antisense)

Primer combinations for outer and inner primer pairs

forward primer	reverse primer
Outer primer pairs:	
mgag1	mgag5
mgag2	mgag5
Inner primer pairs:	
mgag2	mgag4
mgag3	mgag4

Figure I-12. Sequences related to the *env* gene of MMTV.

Mouse	TCCCTTCCCT CGCCTAGTGT AGATCAGTCA GATCAGATTA AAAGCAAAAA
Human 1	----- ----- ----- T ----- -----
Human 2	----- ----- ----- ----- ----- A -----
Human 3	----- ----- A ----- ----- A ----- A -----
Rhesus 1	----- ----- ----- ----- ----- A -----
Rhesus 2	----- ----- ----- ----- ----- A -----
Cat 1	----- ----- ----- ----- ----- A -----
Cat 2	----- ----- ----- ----- ----- A -----
Mouse	GGATCTATTG GGAAATTATA CTCCCCCTGT CAATAAAGAG GTTCATCGAT
Human 1	----- ----- ----- ----- -----
Human 2	----- ----- ----- ----- -----
Human 3	----- C ----- . ----- -----
Rhesus 1	----- ----- ----- ----- -----
Rhesus 2	----- ----- ----- ----- -----
Cat 1	----- C ----- ----- -----
Cat 2	----- C ----- ----- -----
Mouse	GGTATGAAGC AGGATGGGTA GAACCTACAT GGTTCTGGGA AAATTCTCCT
Human 1	----- ----- ----- ----- -----
Human 2	----- ----- ----- T ----- -----
Human 3	----- ----- ----- T ----- -----
Rhesus 1	----- ----- ----- T ----- -----
Rhesus 2	----- ----- ----- T ----- -----
Cat 1	----- ----- ----- ----- -----
Cat 2	----- ----- ----- ----- -----
Mouse	AAGGATCCCA ATGATAGAGA TTTTACTGCT CTAGTTCCCC ATACAGAATT
Human 1	----- ----- ----- ----- -----
Human 2	----- ----- ----- ----- -----
Human 3	----- ----- ----- ----- -----
Rhesus 1	----- A ----- ----- ----- -----
Rhesus 2	----- A ----- ----- ----- -----
Cat 1	----- ----- ----- T ----- -----
Cat 2	----- ----- ----- ----- -----
Mouse	GTTTCGCTTA GTTGCAGCCT CAAGATATCT TATTCTCAAAGGCCAGGAT
Human 1	----- ----- ----- ----- -----
Human 2	----- ----- ----- C ----- ----- A -----
Human 3	----- ----- ----- C ----- ----- A -----
Rhesus 1	----- ----- ----- C ----- ----- A -----
Rhesus 2	----- ----- ----- C ----- ----- A -----
Cat 1	----- ----- ----- C ----- ----- A -----
Cat 2	----- ----- ----- C ----- ----- A -----

Figure I-12. continue

Mouse	TTCAAGAAC	TGACATGATT	CCTACATCTG	CCTGTGTTAC	TTACCCTTAT
Human 1	-----	-----	-----	-----	-----
Human 2	-----	-----G	-----	-----	-----
Human 3	-----	-----G	-----	-----	-----
Rhesus 1	-----	-----G	-----	-----	-----
Rhesus 2	-----	-----G	-----	-----	-----
Cat 1	-----	-----A-G	-----	-----	-----
Cat 2	-----	-----A-G	-----	-----	-----
Mouse	GCCATATTAT	TAGGATTACC	TCAGCTAATA	GATATAGAGA	AAAGAGGATC
Human 1	-----	-----	-----	-----A	-----
Human 2	-----	-----	-----	-----	-----
Human 3	-----	-----	-----	-----G	-----
Rhesus 1	-----	-----	-----	-----	-----
Rhesus 2	-----	-----	-----	-----	-----
Cat 1	-----	-----	-----	-----	-----
Cat 2	-----	-----A	-----	-----	-----
Mouse	TACTTTTCAT	ATTCCTGTT	CTTCTTGTAG	ATTGACTAAT	TGTTTAGATT
Human 1	-----	-----	-----	-----	-----
Human 2	-----	-----	-----T	-----	-----
Human 3	-----	-----	-----	-----	-----C
Rhesus 1	-----	-----	-----	-----	-----
Rhesus 2	-----	-----	-----CC	-----	-----
Cat 1	-----	-----	-----	-----	-----
Cat 2	-----	-----	-----	-----	-----
Mouse	CTTCTGCCTA	CGACTATGCA	GCGATCATAG	TCAAGAGGCC	GCCATACGTG
Human 1	-----	-----	-----	-----A-G	-----T-----
Human 2	-----	-----	-----	-----	-----
Human 3	-----	-----	-----	-----	-----
Rhesus 1	-----	-----	-----	-----	-----
Rhesus 2	-----	-----	-----	-----	-----
Cat 1	-----	-----	-----	-----	-----
Cat 2	-----	-----	-----	-----	-----
Mouse	CTGCTACCTG	T			
Human 1	-----	-----			
Human 2	-----	-----			
Human 3	-----	-----			
Rhesus 1	-----	-----			
Rhesus 2	-----	-----			
Cat 1	-----	-----			
Cat 2	-----	-----			

Figure I-12. Sequences related to the *env* gene of MMTV.

Human 1 = **human breast cancer**; Human 2 = **human colon carcinoma (Z14)**;
 Human 3 = **human renal cell carcinoma (Z7)**; Rhesus 1 & 2 = **Rhesus macaque (Z15)**, two amplifications reactions; Cat 1 & 2 = **kitten thymus (Z9)**, two amplification reactions.

Mouse	ATGATGCCGA	GAGGAGAAGG	GTCAGATATA	TTGATCAAGC	AATTGGCATG
Cat	-----	-----	-----	A-----	G-A
Mouse	GGAAAATGCA	AATTCAATTGT	GTCAGGATCT	CATCCGCCCA	ATACGAAAAA CAGG
Cat	AA-----	-----	C-A-----	T-----T-----	-----

Figure I-13. Sequence related to the *gag* gene of MMTV, isolated from a cat.

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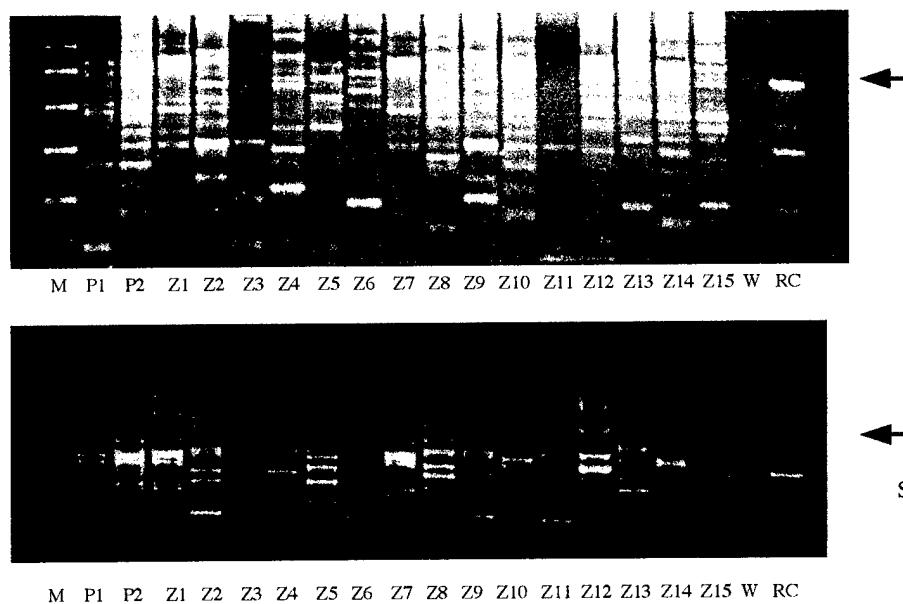
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Figure I-1.

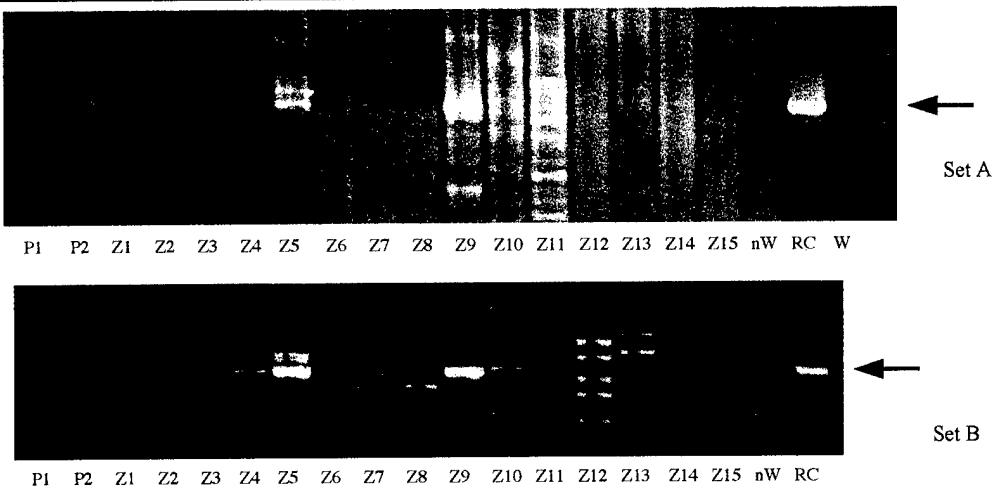


First round amplification with mmtv-1 and mmtv-4 primers.

"Set A" and "Set B" represent two sets of PCR assembled at different times. Arrow indicates expected size.

M= molecular markers (50, 100, 300, 500, 750, 1000 bps); P1= human peripheral blood lymphocytes #1, P2= human peripheral blood lymphocytes #2, Z1= human (squamous cell carcinoma), Z2= cat (normal kidney), Z3= lambda, Z4= dog (spleen), Z5= mouse (Balb/c, liver), Z6= sea urchin, Z7= human (renal cell carcinoma), Z8= rat (embryo fibroblasts), Z9= cat (normal kitten thymus), Z10= human (sperm), Z11= cat (normal spleen), Z12= chicken (thymus), Z13= snake (liver), Z14= human (colon carcinoma), Z15= Rhesus macaque, W= water lane control introduced in first round of PCR, RC= mouse recombinant control introduced in first round of PCR.

Figure I-2.

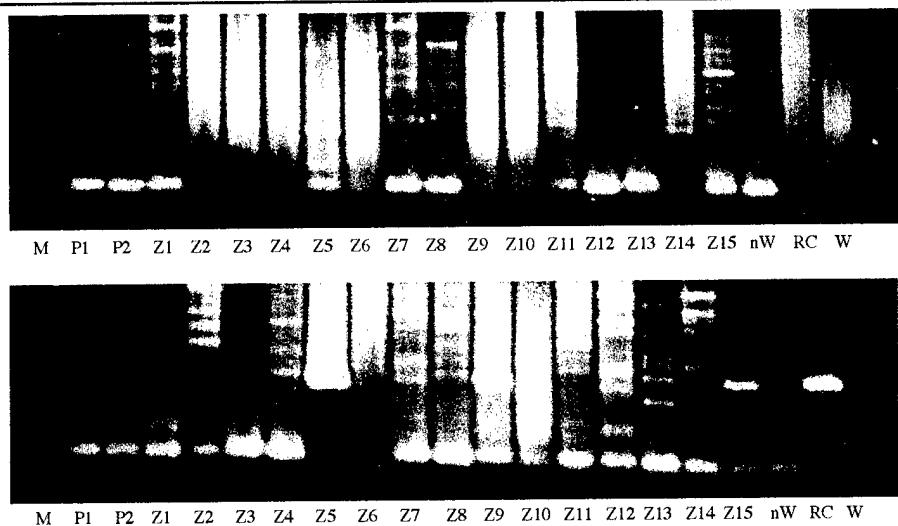


Second round amplification with mmtv-L5 and mmtv-L3 primers.

"Set A" and "Set B" represent two sets of PCR assembled at different times. Arrow indicates expected size.

M= molecular markers (50, 100, 300, 500, 750, 1000 bps); P1= human peripheral blood lymphocytes #1, P2= human peripheral blood lymphocytes #2, Z1= human (squamous cell carcinoma), Z2= cat (normal kidney), Z3= lambda, Z4= dog (spleen), Z5= mouse (Balb/c, liver), Z6= sea urchin, Z7= human (renal cell carcinoma), Z8= rat (embryo fibroblasts), Z9= cat (normal kitten thymus), Z10= human (sperm), Z11= cat (normal spleen), Z12= chicken (thymus), Z13= snake (liver), Z14= human (colon carcinoma), Z15= Rhesus macaque, W= water lane control introduced in first round of PCR ("template" for "nested water" in second round), nW= water lane control introduced in second round of PCR, i.e. nested water, RC= mouse recombinant control introduced in first round of PCR.

Figure I-3.

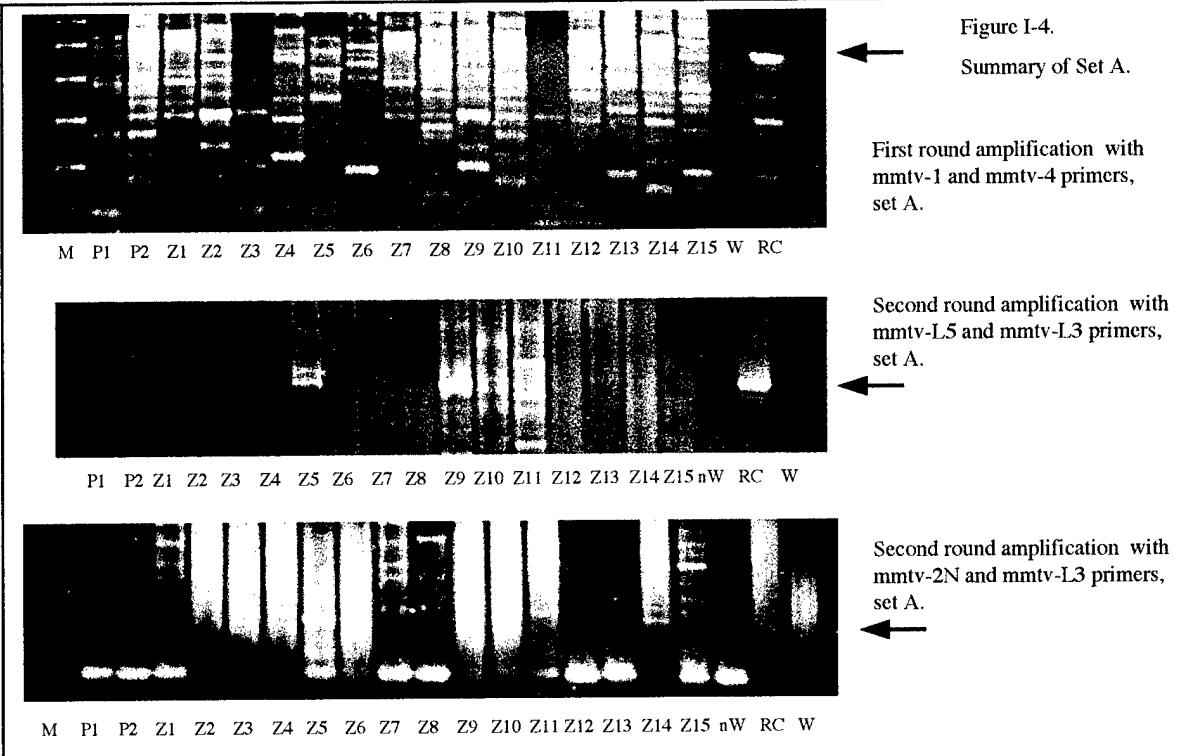


Second round amplification with mmtv-2N and mmtv-L3 primers.

"Set A" and "Set B" represent two sets of PCR assembled at different times. Arrow indicates expected size.

M= molecular markers (50, 100, 300, 500, 750, 1000 bps); P1= human peripheral blood lymphocytes #1, P2= human peripheral blood lymphocytes #2, Z1= human (squamous cell carcinoma), Z2= cat (normal kidney), Z3= lambda, Z4= dog (spleen), Z5= mouse (Balb/c, liver), Z6= sea urchin, Z7= human (renal cell carcinoma), Z8= rat (embryo fibroblasts), Z9= cat (normal kitten thymus), Z10= human (sperm), Z11= cat (normal spleen), Z12= chicken (thymus), Z13= snake (liver), Z14= human (colon carcinoma), Z15= Rhesus macaque, W= water lane control introduced in first round of PCR ("template" for "nested water" in second round), nW= water lane control introduced in second round of PCR, i.e. nested water, RC= mouse recombinant control introduced in first round of PCR.

Figure I-4.
Summary of Set A.



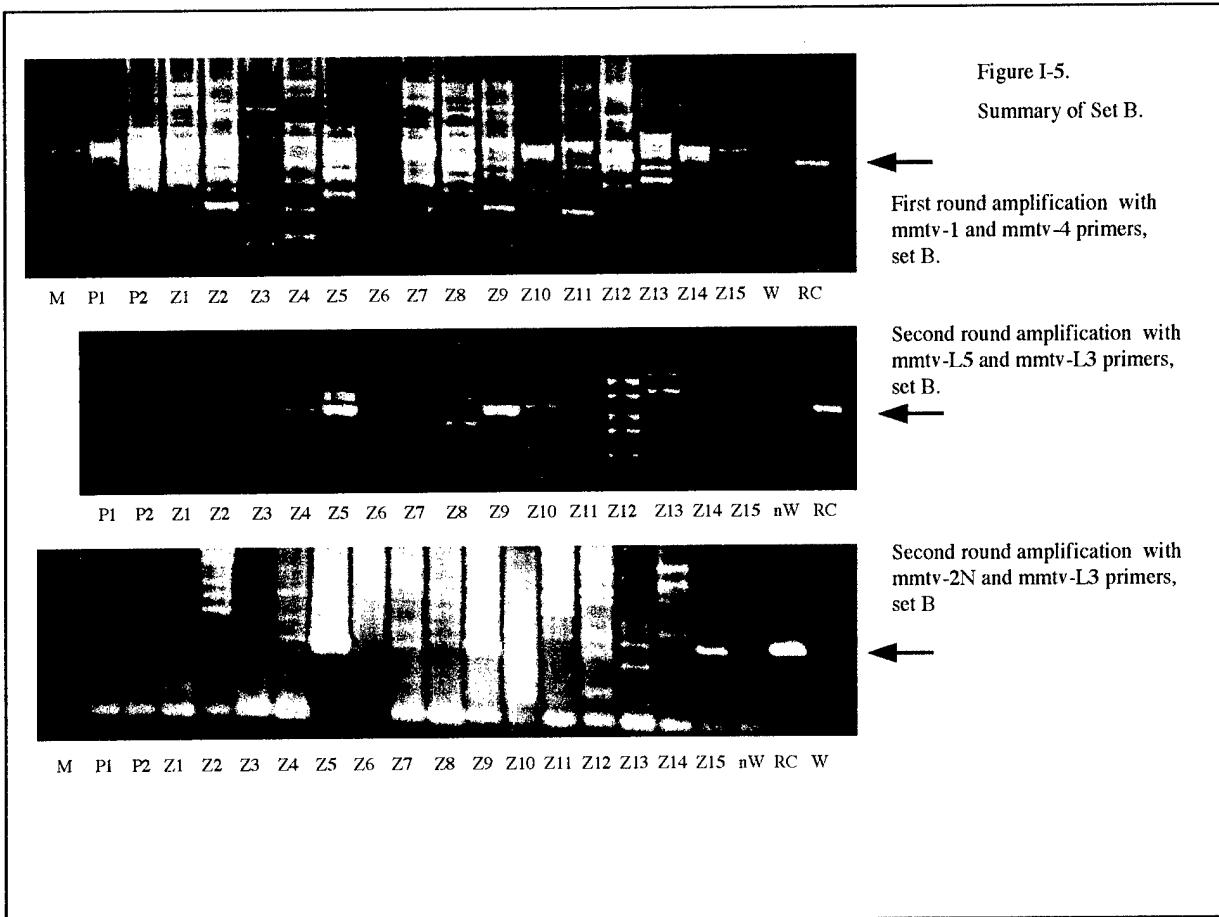
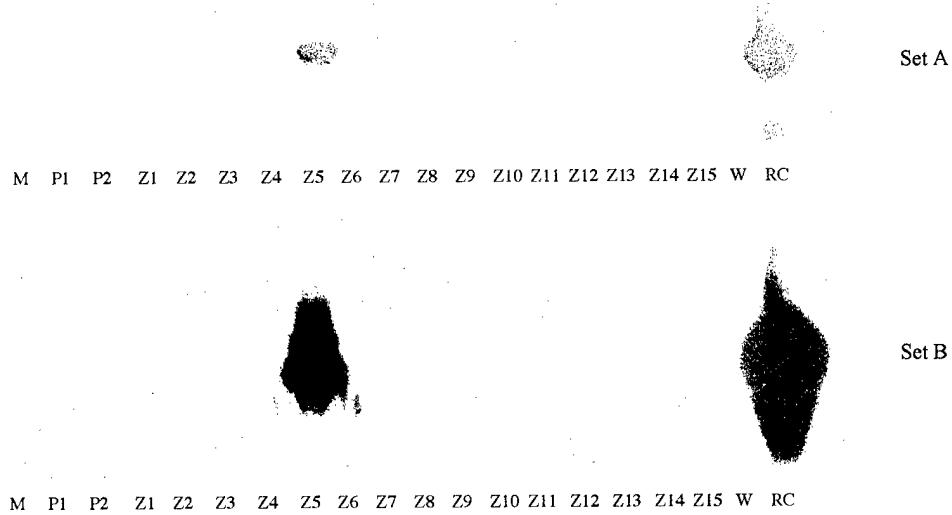


Figure I-6.

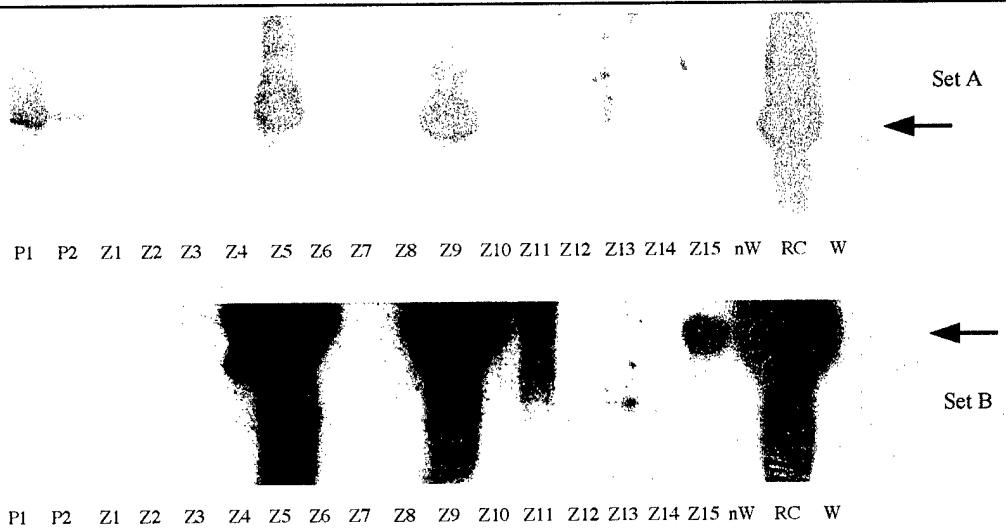


PCR blot of first round amplification with mmmtv-1 and mmmtv-4 primers.

The only positive sample is Z5= mouse (Balb/c, liver).

W= water lane control introduced in first round of PCR, RC= mouse recombinant control introduced in first round of PCR.

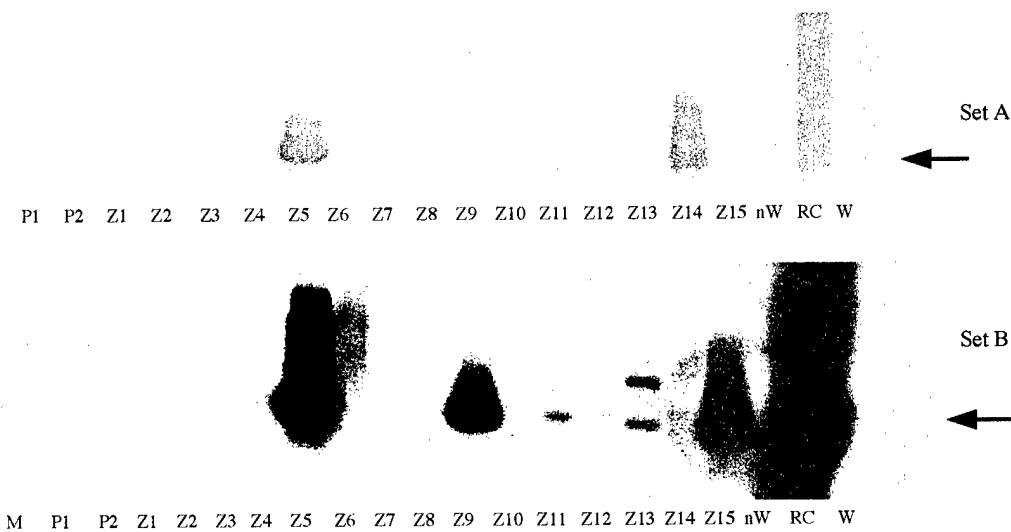
Figure I-7.



PCR blot of second round amplification with mmtv-L5 and mmtv-L3 primers.

In set A, variably positive signal seen in the following samples: P1= human peripheral blood lymphocytes #1, P2= human peripheral blood lymphocytes #2, Z5= mouse (Balb/c, liver), Z9= cat (normal kitten thymus), Z13= snake (liver).
In set B, variably positive signal seen in the following samples: Z5= mouse (Balb/c, liver), Z9= cat (normal kitten thymus), Z11= cat (spleen), Z15= Rhesus macaque.
W= water lane control introduced in first round of PCR ("template" for "nested water" in second round), nW= water lane control introduced in second round of PCR, i.e. nested water, RC= mouse recombinant control introduced in first round of PCR.

Figure I-8.



PCR blot of second round amplification with mmtv-2N and mmtv-L3 primers.

In set A, positive samples are: Z5= mouse (Balb/c, liver), Z14= human (colon carcinoma).
In set B, variably positive signal seen in the following samples: Z5= mouse (Balb/c, liver), Z9= cat (normal kitten thymus), Z11= cat (spleen), Z13= snake (liver) and Z15= Rhesus macaque.
W= water lane control introduced in first round of PCR ("template" for "nested water" in second round), nW= water lane control introduced in second round of PCR, i.e. nested water, RC= mouse recombinant control introduced in first round of PCR.

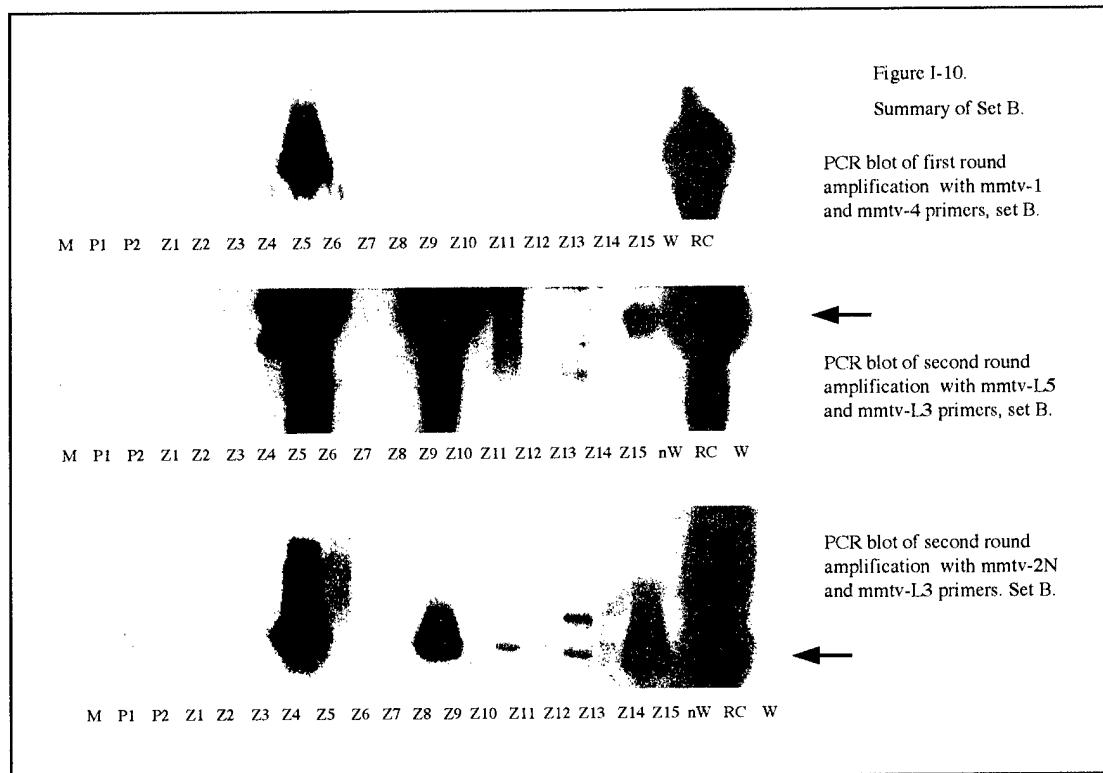
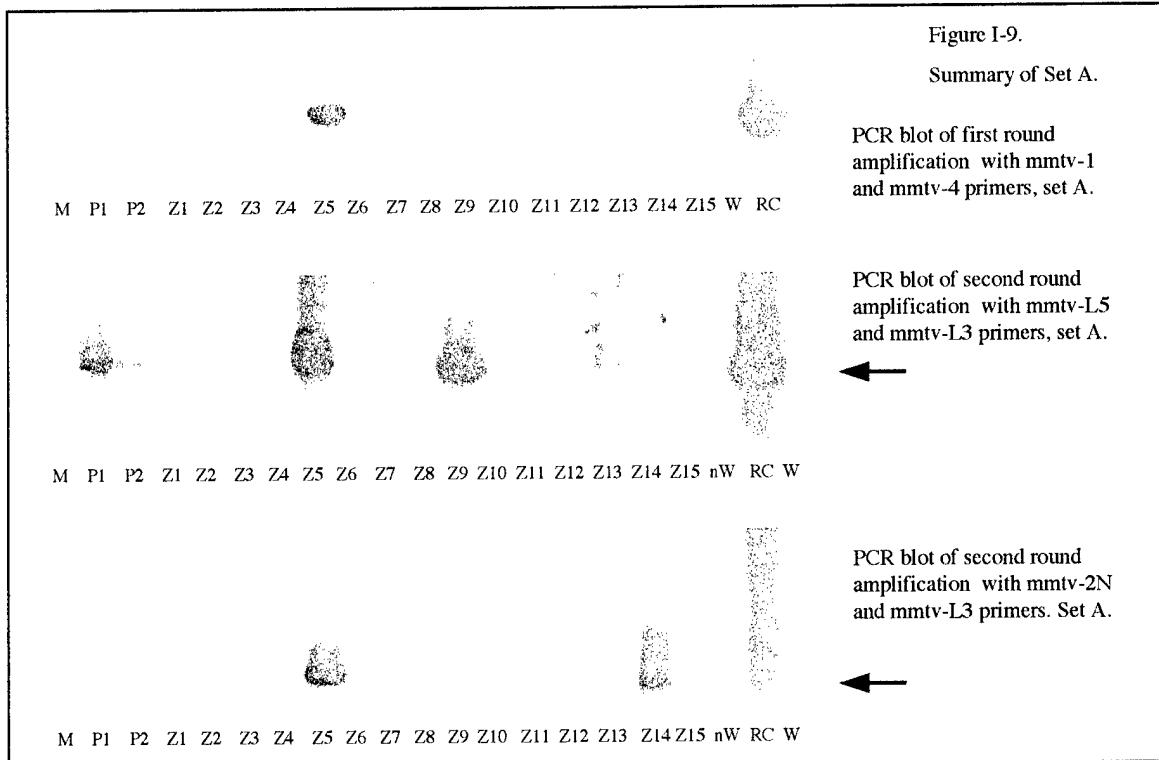
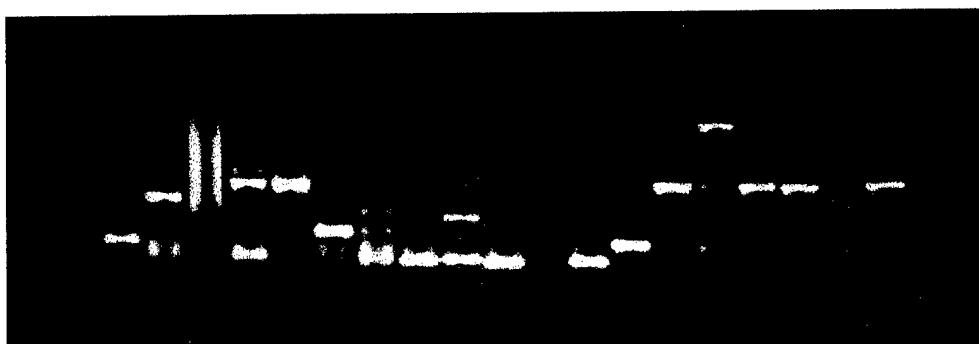


Figure I-11.



* * * * W RC

Representative set of colony-screen by PCR from a positive sample with mmtv-L5 and mmtv-L3 primers. Asterisks indicate colonies that amplify band of expected size. Smaller or larger bands represent artifacts. W= template-free "water control"; RC= mouse recombinant control.

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HUMAN MOUSE MAMMARY TUMOR VIRUS: AN UPDATE

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Variations of several genes, including BRCA-1 and BRCA-2, can result in greatly increased risks for development of breast cancer (BC). However, defects in known BC susceptibility genes can account for only about 5% of BC, the so-called familial cases. The possibility that a virus is etiologically involved in sporadically occurring BC has not been eliminated, and indeed is consistent with a large body of scientific evidence accumulated over several decades.

Mouse mammary tumor virus (MMTV), now classified in the Betaretroviridae, is the prototype of slow-transforming retroviruses. MMTV has been definitively shown to cause BC in mice. MMTV is transmitted both in the germline as endogenous proviruses and exogenously as infectious virions. Vertically transmitted endogenous MMTV proviruses follow patterns of Mendelian inheritance, as other sequences in the genome (Cohen et al., 1982; Cohen and Varmus, 1979; Cohen and Varmus, 1980; Traina-Dorge and Cohen, 1983; Traina-Dorge et al., 1985; Varmus et al., 1980; Varmus et al., 1978) Horizontal transmission of MMTV typically occurs by infection of mouse pups by MMTV virions present in the milk of infected dams. MMTV proviral LTR elements direct steroid hormone-dependent transactivation of various cellular oncogenes including *Wnt*, *Fgf* and *Int* thereby promoting clonal expansion of tumor cells (Jakobovits et al., 1986; Nusse, 1988; Nusse et al., 1991; Shackleford et al., 1993; Shackleford and Varmus, 1988) (Fig. 2). For productive persistent infection and completion of its replication cycle, MMTV must contain a superantigen and interact with a functional host immune system (Coffin, 1992; Golovkina et al., 1995; Luther et al., 1997) (Fig. 2).

More than thirty unique proviral integration sites for endogenous MMTV have been identified. However, some wild mice do not carry any endogenous MMTV proviruses suggesting that the many endogenous MMTV proviruses are relatively recent additions to the mouse

genome(Cohen et al., 1982; Cohen and Varmus, 1979). MMTV appears to have entered the germline of certain mice (but not others) on multiple occasions after the evolutionary splits among the various species and subspecies of the genus *Mus*. Certain endogenous MMTV can be activated by hormones to form infectious virions capable of inducing mammary carcinomas after long latency periods.

Sequences with very high similarity to those of MMTV have been detected in DNA of human BC tissue (An et al., 2001; Pogo and et al., 1997; Pogo et al., 1999; Wang et al., 1998; Wang et al., 1995; Wang et al., 2001). Sequences 95 - 99% similar to MMTV *env* were amplified by PCR in 121 (38.5%) of 314 unselected breast cancer tumor samples. The MMTV-like sequences were detected in only 2 (1.8%) of 107 breast specimens from reduction mammoplasties and in 0/80 samples from normal tissues or non-breast tumors. The MMTV-*env* like RNA was expressed (as determined by RT-PCR) in 66% of DNA PCR positive breast tumors (Wang et al., 1998). A complete 9.9 kb provirus with 94% similarity to MMTV was detected in 2 breast tumors (Liu et al., 2001). FISH revealed integration at several sites in BC DNA, but not normal breast cells. These authors suggested the existence of a human mammary tumor virus (HMTV) that is spread by the exogenous route of infection (horizontal transmission). These and other investigators' attempts to amplify other regions of MMTV-related viruses from genomic DNA or cDNA of subjects free of BC yielded HERV sequences (such as HERV-K10) with only about 60% homology to MMTV. Thus, BC tissues are the only tissues in which sequences that are highly similar to those of MMTV have been found, while normal breast and other tissues appeared to be negative.

In contrast to these results, we have found sequences closely related to MMTV by PCR and a sensitive blotting technique, not only in breast tumors, but also in the blood of a subset of

healthy controls. Our results suggest that the genomes of a subset (~10%) of humans contain an integrated retrovirus(es) that is/are closely related to MMTV.

We have PCR amplified sequences highly similar (>95%) to the MMTV *env* gene from human DNA samples, including subsets of both BC tissue and nonBC tissues. We found the MMTV-related sequences by PCR and a sensitive blotting technique not only in breast tumors (Fig. 1), but also in the blood of a subset of healthy controls (Fig. 2), and systemic lupus erythematosus (SLE) patients without breast cancer (not shown). Our results differ from those of Wang and coworkers (1995) who, with few exceptions, were able to detect MMTV-like sequences only in breast tumors. The sequences from human DNA were distinct from the MMTV sequences used as controls in these PCR reactions indicating that our results are not simply due to contamination (Figs. 3 and 4). A ribonuclease protection assay was used to confirm these results using a non-PCR based technique to determine that the majority of the PCR positive BC tissues, but none of the PCR negative tissues, expressed this sequence at the mRNA level. We have also sequenced many of the products from our PCR reactions (Figs. 5). PCR contamination is an unlikely explanation for our results. MMTV *env*-like sequences from different individuals derived in the same PCR run were distinct from each other (Fig. 6). This result indicates the lack of an ubiquitous PCR contaminant that would have produced a more consistent sequence that should have been identical (or nearly so) in the various reaction tubes. Furthermore DNA of individual subjects produced internally consistent MMTV *env*-like sequences from PCR run to PCR run (Fig. 6). The variations within the MMTV-related sequences from a given patient may represent a low number of Taq errors, but are also suggestive of variations expected of a replicating retrovirus (RT errors).

In addition to mice and humans, we amplified sequences from nonBC genomic DNA of a subset of rhesus macaques distinct from, but highly related to, MMTV *env* (Fig. 3). A MMTV *env*-related sequence was also cloned from the DNA of 1 of 3 cats examined (Fig. 3), but a variety of DNAs from other sources (lambda phage, spider, cockroach, sea urchin, frog, chicken, and dog) were negative (not shown). We also amplified from cat DNA a sequence approximately 90% similar to the MMTV group antigen gene (*gag*) (Fig. 7). Human *gag* sequences were also amplified by these primers, but have not yet been cloned and sequenced. Two of 18 rhesus macaques(11%) had MMTV-related sequences. These results indicate that vertebrate species other than mice, including some humans and rhesus macaques, can contain sequences closely related to MMTV, and suggest that it may be feasible to establish a primate model to explore the role of these sequences in BC.

The discovery of MMTV prompted many investigators to explore a retroviral etiology for BC in humans (Chopra and Feller, 1969; Dmochowski, Seman, and Gallager, 1969; Feller and Chopra, 1971; Keydar et al., 1984; Litvinov and Golovkina, 1989). Data collected over the past six decades has suggested the existence of a human homologue of MMTV. In 1971, Moore and associates reported that 60% of human milk samples from BC patients contain B-type particles indistinguishable from MMTV by electron microscopy, compared to 5% of the general population (Moore, 1971). These investigators also reported that 39% of Parsi women of India, an inbred population with a two-fold increased incidence of BC, had B-type particles in their milk (Das et al., 1972). Numerous investigators have examined serum and breast milk for the presence of antibodies reactive with MMTV. Most of these studies were performed in the pre-AIDS era, prior to the advent of highly sensitive and specific techniques for detecting anti-retroviral antibodies made necessary for detection of HIV antibodies in donated blood. Despite the rather insensitive assays employed, most investigators have found that the majority of BC patients produce antibodies in serum or breast milk capable of neutralizing MMTV infectivity or reacting with MMTV proteins or particles in infected cells (Litvinov et al., 1986; Litvinov and Golovkina, 1989; Shoenfeld et al., 1987; Sibal and Rubin, 1972). In nearly all previous immunological studies performed, a percentage of women (10-25%) without breast cancer also produced MMTV reactive antibodies. This result is consistent with the finding of MMTV-like particles in milk of healthy women and our observations (Fig. 4) that a subset of humans are carriers of MMTV-related sequences.

Despite the numerous electron microscopic, biochemical and immunological studies on human breast carcinoma tissue, milk, patients' sera, and breast carcinoma cell lines suggesting the existence of a human homologue of MMTV, proof that such an agent exists has remained

elusive. Most authors have dismissed the importance of prior studies purporting to show evidence of a human homologue of MMTV because of the presence of numerous human endogenous retroviruses (HERVs) (Callahan et al., 1982; May and Westley, 1986; May et al., 1983; Ono et al., 1986). There are about 50,000 HERVs or HERV-related sequences in the human genome, some of which have been shown to have up to 60% homology to MMTV. In this regard, it is important to note that seroreactivity to HERV-K10, to this point the HERV most closely related to MMTV, cannot account for MMTV-reactive antibodies present in the sera of breast cancer patients and the smaller number of healthy individuals (Vogetseeder et al., 1995). Furthermore, we believe that the presence of these MMTV-related sequences is precisely the reason that human homologues of MMTV have not previously been demonstrated conclusively by molecular techniques. The presence of these related, but distinct, sequences could have obscured the detection of more closely related sequences by prior investigators who used less sensitive techniques, such as Southern blotting.

The molecular genetic interactions between MMTV, the immune system of its murine host, and the breast and other hormonally-sensitive cells malignantly transformed by this retrovirus have been extensively studied. This extensive body of research can be exploited in the current proposal which describes the discovery of MMTV-like sequences in humans, rhesus macaques and other species.

Fig. 1. Amplification of sequences related to MMTV from human breast cancer tissue.

Panel A: DNA was extracted from human breast tumors (kindly provided by Michael Press, M.D., USC, Los Angeles or Derrick Beech M.D., TMC/UT Memphis) and PCR was performed using primers specific for the human MMTV *env*-related gene. PCR products were transferred to nitrocellulose by blotting and MMTV-related products were detected by hybridization to a 1.8 kb MMTV *env* probe. lane a: nonradioactive markers, not shown; lanes b-d, f-h, j: breast tumor DNA; lanes e and i: no DNA; lane k: water control; lane l positive control: MMTV *env* fragment cloned in pBluescript. Panel B: As a test for the integrity of the DNA from the clinical samples we amplified HERV-3 proviral DNA, a single copy human endogenous retrovirus using PCR conditions developed by Griffiths *et al.* (Griffiths *et al.*, 1997). Ethidium bromide detection (markers are visible in lane a). Same samples as Panel A except lane l: positive control, HERV3 *pol* fragment cloned in pBluescript. Visible bands were present in lanes c and g, but do not copy well.

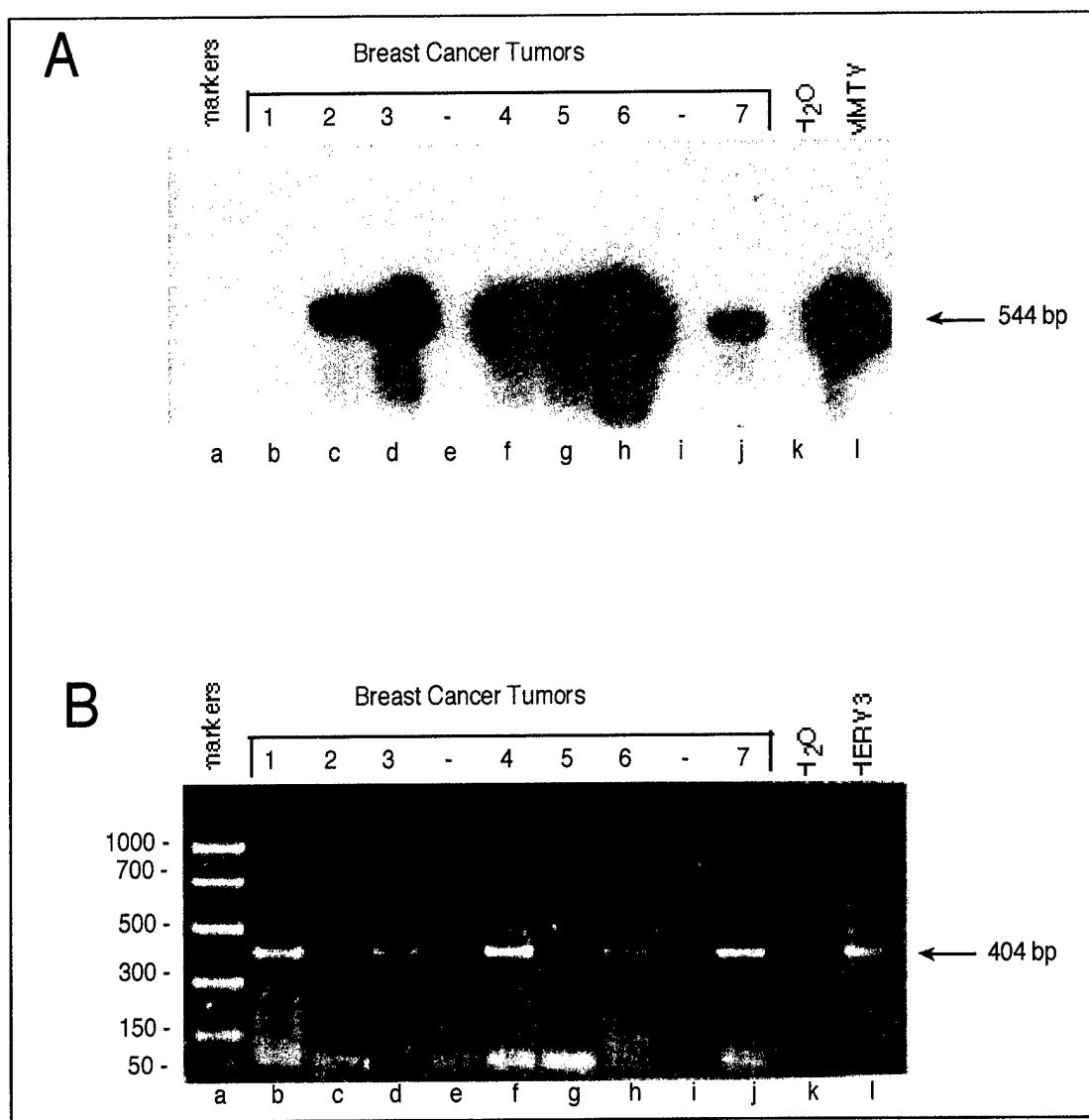
Fig2. Amplification of sequences related to MMTV from the blood of healthy controls.

Panel A: DNA was extracted from whole blood of healthy control subjects and PCR was performed using primers specific for the human MMTV *env*-related gene and PCR products were detected by southern hybridization as described in the legend of Fig. 5. lane a: markers; lanes b-k: DNA from whole blood of healthy controls; lane l: water control; lane m positive control: MMTV *env* fragment in pBluescript. Panel B: PCR amplification of HERV-3. Ethidium bromide detection (markers are visible in lane a). Same samples as Panel A except lane m: positive control, HERV3 *pol* fragment in pBluescript.

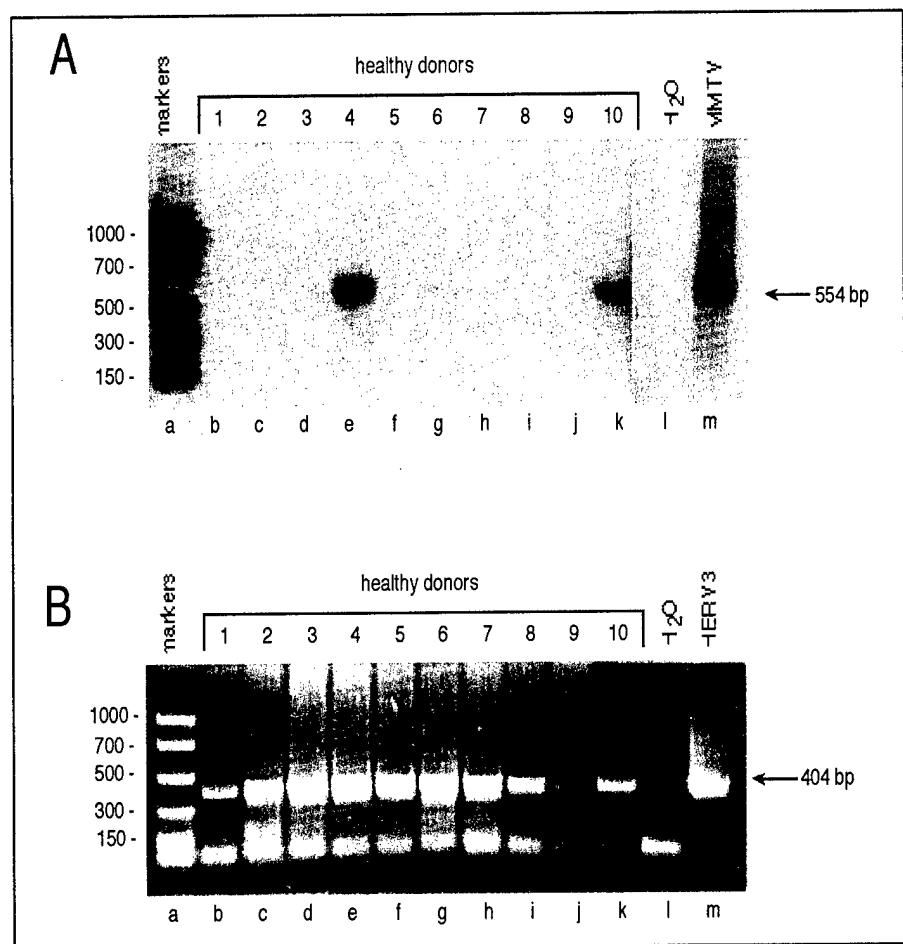
Fig. 3. Sequences related to the env gene of MMTV in humans, rhesus macaque and cat.

PCR products of MMTV-related sequences of humans, cats and rhesus macaques were cloned directly into the pCRII vector of the TA Cloning Kit (Invitrogen). Human 1 is from tumor 7 in Fig. 1. The MMTV sequence was obtained from the cell line (C3H) used as the source of control DNA. Plasmid DNA was prepared from colonies with an appropriate sized DNA insert by the MAXI Prep procedure (Qiagen). Products were manually sequenced (Sequenase I 2.0, USB) using T7 primers and M13 reverse primers that bind to sequences that flank the insertion site in this version of pCRII or sequenced with an automated sequencer.

Fig. 4. Variation among MMTV-related envelope sequences. These depictions represent the MMTV-related *env* sequences of two SLE patients (SLE 1 and 2) and a healthy subject (human 3). Sequences were determined by automated sequencing and confirmed by sequencing both the forward and reverse DNA strands. Vertical bars represent nucleotide differences from the laboratory strain of MMTV used as a positive PCR control in these reactions. Gaps represent single base pair deletions present in both forward and reverse strands. The human sequences were obtained in various PCR runs (A-C) on different days months apart. Each of the three representative subjects gave internally consistent sequences with only subtle variations from PCR run to PCR run. These results indicate a lack of laboratory PCR contamination that would have been evident by the finding of identical sequences for the PCR products derived in the various reactions.



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Mouse TCCCTTCCCT CGCCTAGTGT AGATCAGTCA GATCAGATTA AAAGCAAAA GGATCTATTT GGAAATTATA CTCCCCCTGT
Human 1 ----- T ----- ----- ----- ----- -----
Human 2 ----- ----- ----- A ----- ----- -----
Human 3 ----- A A A ----- C . -----
Rhesus 1 ----- ----- A ----- ----- -----
Rhesus 2 ----- ----- A ----- ----- -----
Cat 1 ----- ----- A ----- ----- C -----
Cat 2 ----- ----- A ----- ----- C -----

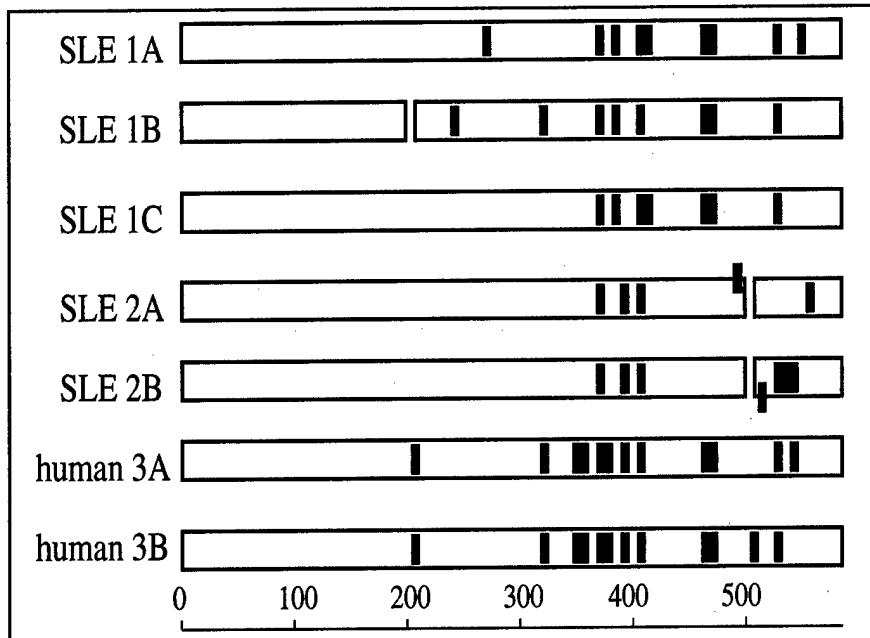
Mouse CAATAAAGAG GTTCATCGAT GGTATGAAGC AGGATGGTA GAACCTACAT GGTTCTGGGA AAATTCTCCT AAGGATCCCA
Human 1 ----- ----- ----- ----- ----- -----
Human 2 ----- ----- ----- T ----- ----- -----
Human 3 ----- ----- ----- T ----- ----- -----
Rhesus 1 ----- ----- ----- T ----- ----- A
Rhesus 2 ----- ----- ----- T ----- ----- A
Cat 1 ----- ----- ----- ----- ----- -----
Cat 2 ----- ----- ----- ----- ----- -----

Mouse ATGATAGAGA TTTTACTGCT CTAGTTCCCC ATACAGAATT GTTTCGCTTA GTTGCAGCCT CAAGATATCT TATTCTCAA
Human 1 ----- ----- ----- ----- ----- -----
Human 2 ----- ----- ----- ----- C ----- -----
Human 3 ----- ----- ----- ----- C ----- -----
Rhesus 1 ----- ----- ----- C ----- -----
Rhesus 2 ----- ----- ----- C ----- -----
Cat 1 ----- T ----- ----- C ----- -----
Cat 2 ----- ----- ----- C ----- -----

Mouse AGGCCAGGAT TTCAAGAAC A TGACATGATT CCTACATCTG CCTGTGTTAC TTACCTTAT GCCATATTAT TAGGATTACC
Human 1 ----- ----- ----- ----- ----- -----
Human 2 -A----- G ----- ----- -----
Human 3 ----- G ----- ----- -----
Rhesus 1-A----- G ----- ----- -----
Rhesus 2-A----- G ----- ----- -----
Cat 1 -A----- A-G ----- ----- -----
Cat 2 -A----- A-G ----- ----- A-----

Mouse TCAGCTAATA GATATAGAGA AAAGAGGATC TACTTTCAT ATTTCCGTGTT CTTCTTGTAG ATTGACTAAT TGTTTAGATT
Human 1 ----- ----- A ----- ----- -----
Human 2 ----- ----- ----- T ----- -----
Human 3 ----- ----- G ----- ----- C-----
Rhesus 1 ----- ----- ----- ----- -----
Rhesus 2 ----- ----- ----- CC -----
Cat 1 ----- ----- ----- ----- -----
Cat 2 ----- ----- ----- ----- -----

Mouse CTTCTGCCTA CGACTATGCA GCGATCATAG TCAAGAGGCC GCCATACGTG CTGCTACCTG T
Human 1 ----- ----- A-G ----- T -----
Human 2 ----- ----- ----- ----- -----
Human 3 ----- ----- ----- ----- -----
Rhesus 1 ----- ----- ----- ----- -----
Rhesus 2 ----- ----- ----- ----- -----
Cat 1 ----- ----- ----- ----- -----
Cat 2 ----- ----- ----- ----- -----



Mouse	ATGATGCCGA	GAGGAGAAGG	GTCAGATATA	TTGATCAAGC	AATTGGCATG
Cat	-----	-----	-----	-----A-----	-----G-A-----
Mouse	GGAAAATGCA	AATTCAATTGT	GTCAGGATCT	CATCCGCCCA	ATACGAAAAA CAGG
Cat	AA-----	-----	-C-A-----	T-----T-----	-----

Figure 7. Sequence related to the MMTV gag gene in cat DNA.

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